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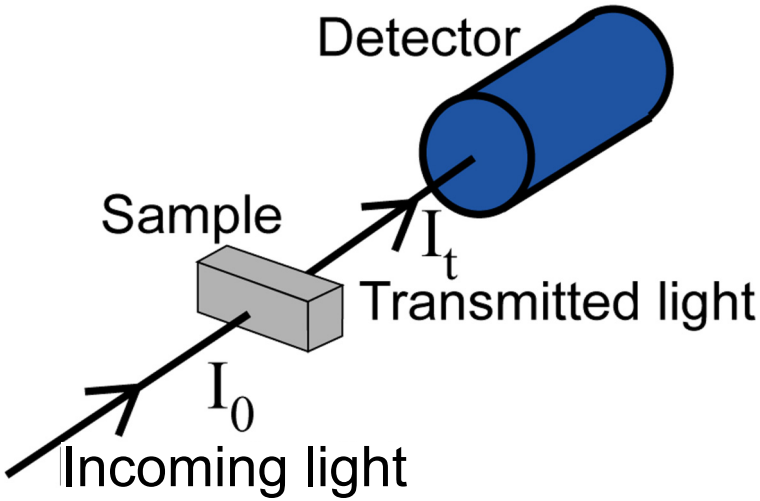
ESC1:
Circular Dichroism:
best practice and data analysis

Lecture 2: Instrumentation

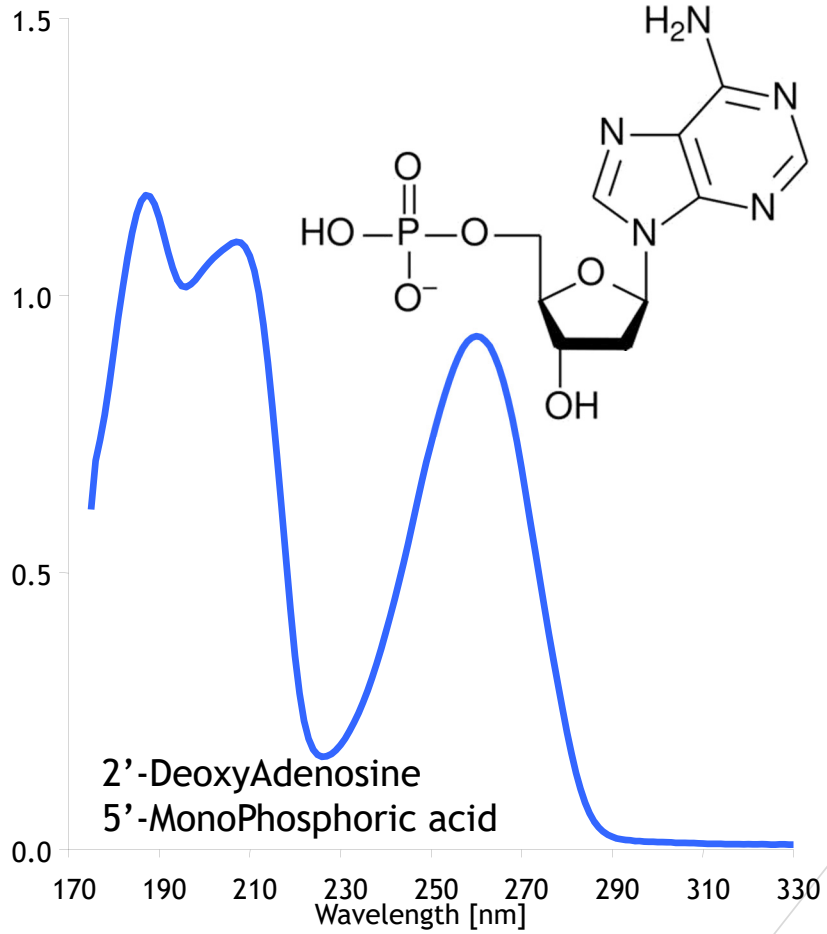
This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

Absorption Spectroscopy



Absorbance:

$A=\log(I_0/I_t)$



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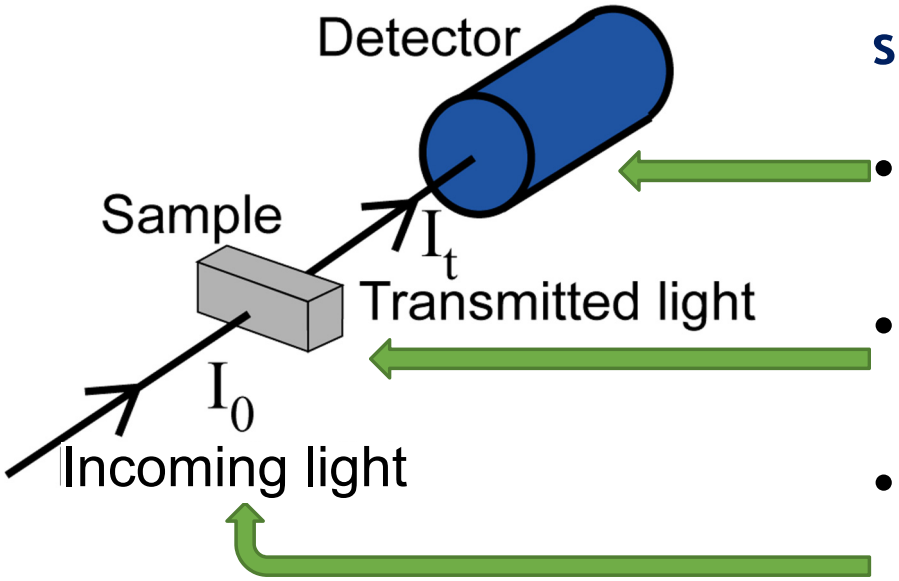
UV-VIS Instrumentation



Shopping list for making UV-VIS spectrometer:

- Detector
- Sample holder
- Light source with monochromatic light

A monochromatic source means a single wavelength of light

→ To make such a source we need a ***lamp*** and ***optics***

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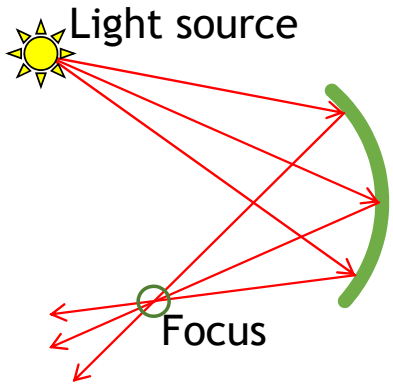
UV-VIS Instrumentation

Mirrors

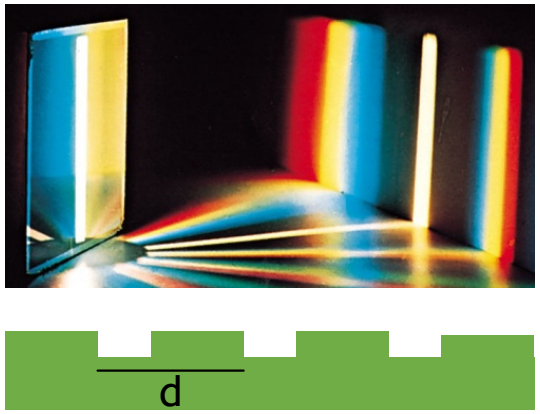
Plane mirrors

Curved mirrors

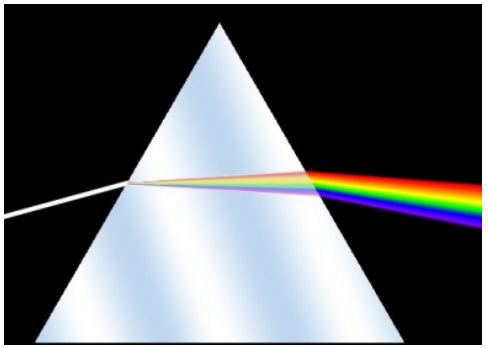


Dispersing elements

Diffraction grating



Prism



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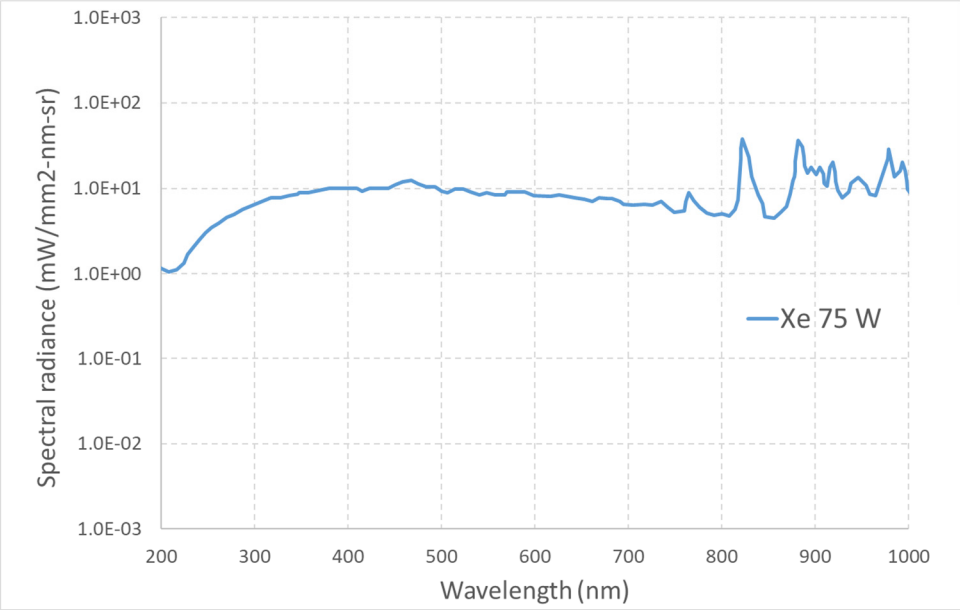
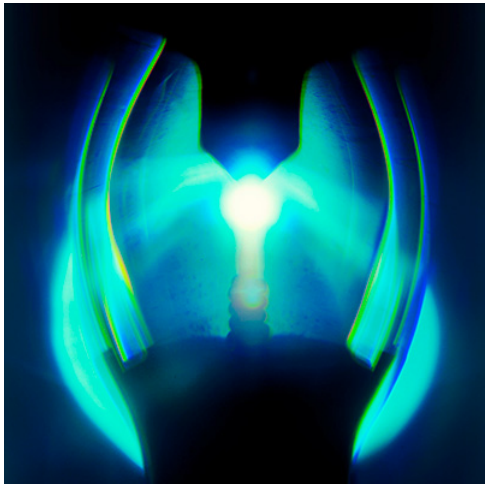
UV-VIS Instrumentation: Light source

Xenon arc lamp





Quite intense UV lamp

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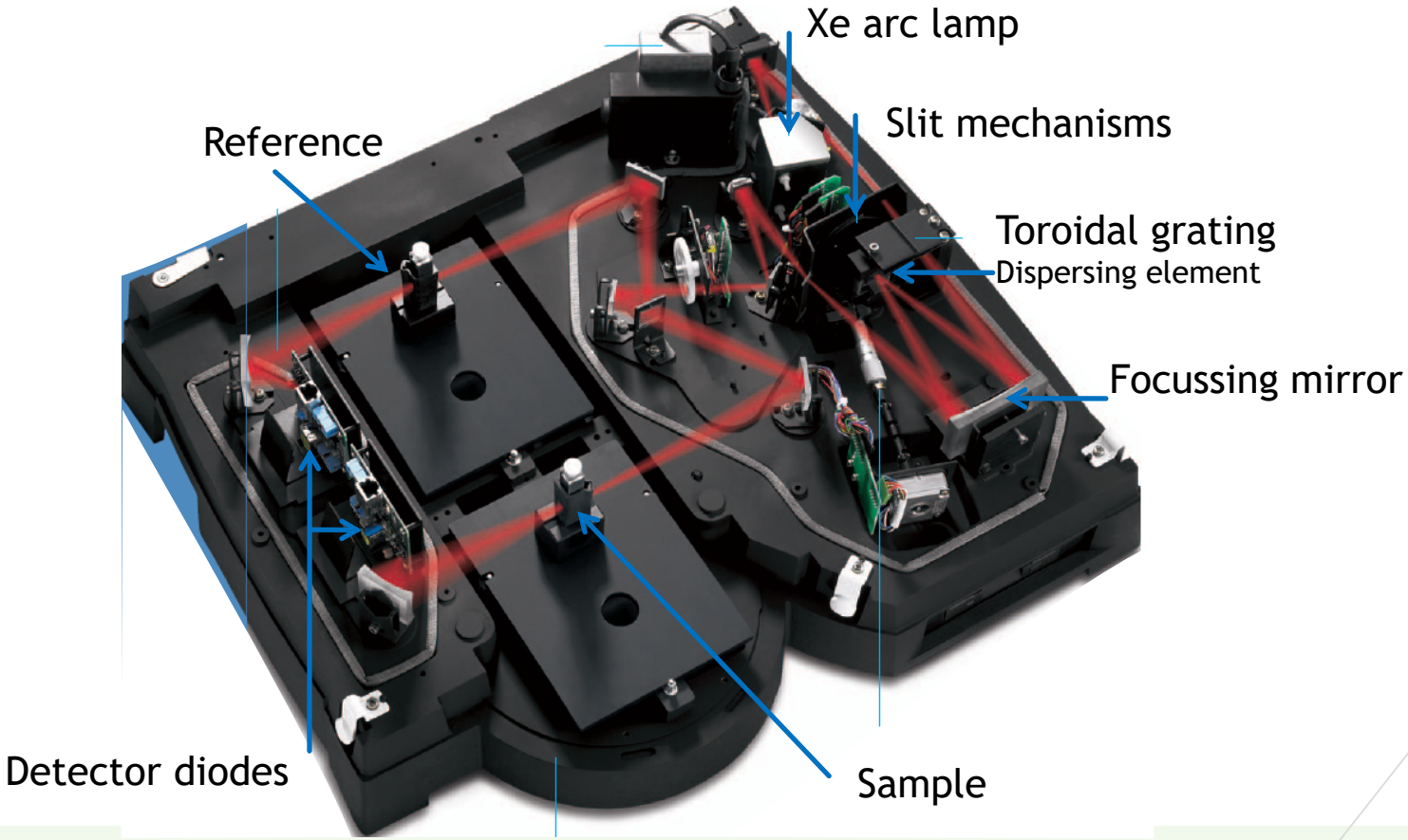
UV-VIS Instrumentation

Conventional UV-VIS instruments



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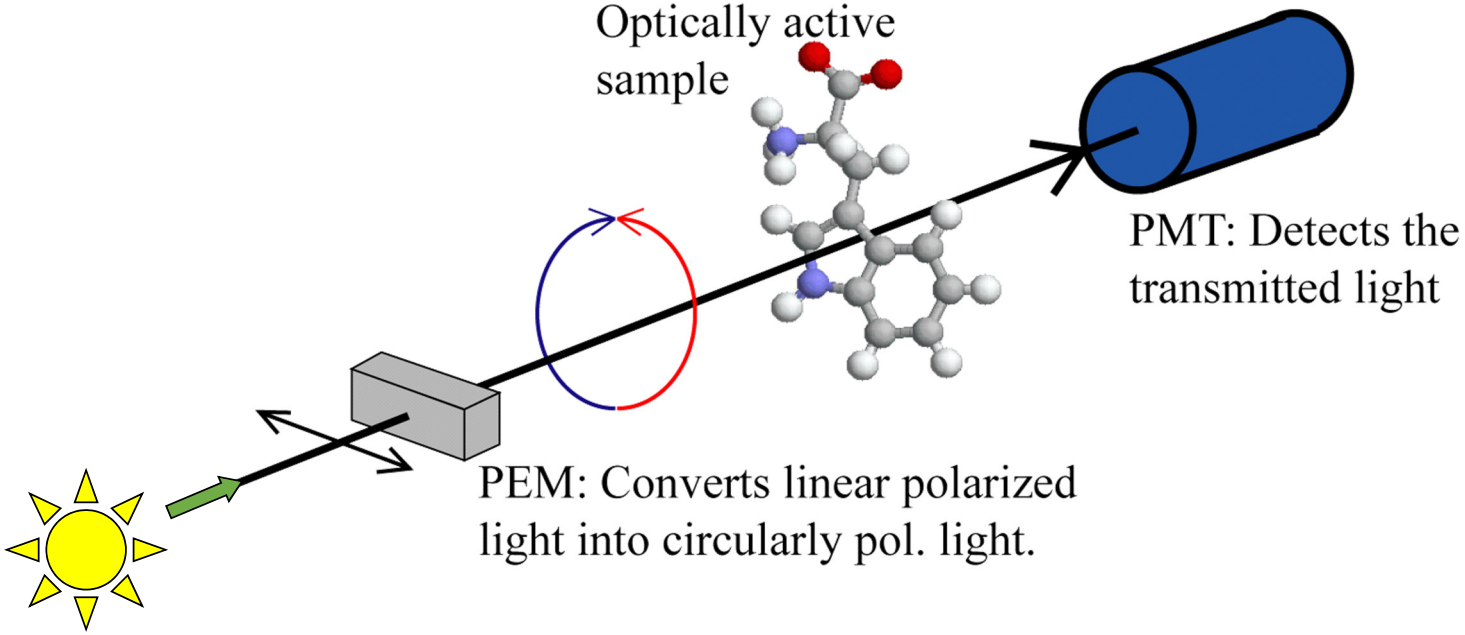
Circular Dichroism

More optical components needed



The CD signal: $\Delta A = A_L - A_R$

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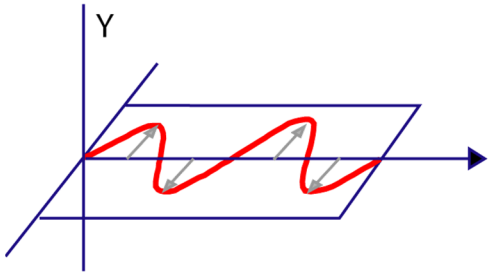
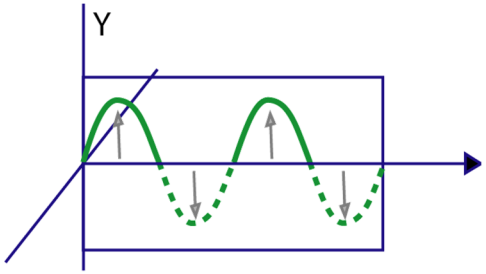
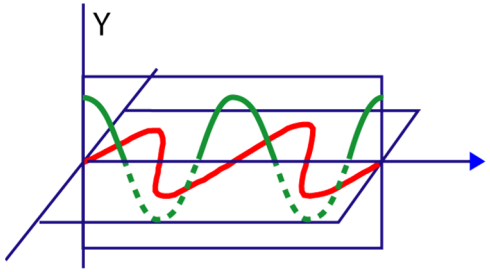
Polarized light



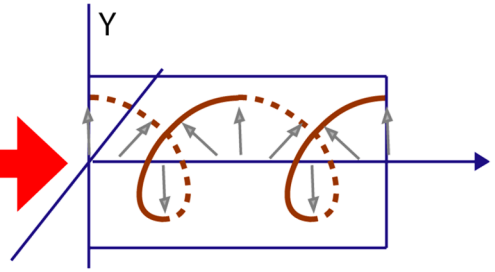
Horizontally pol.



Vertically pol.



Sum of two plane pol.



Circularly pol.

How do we change the polarisation of light ?

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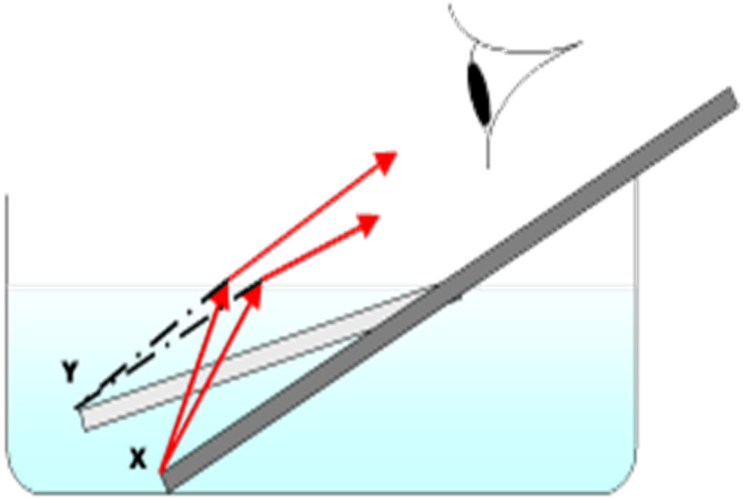
Refraction

Refraction: Light is refracted when travelling from one medium to the next



This is (partly) why it's difficult to catch a fish in water

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Refraction

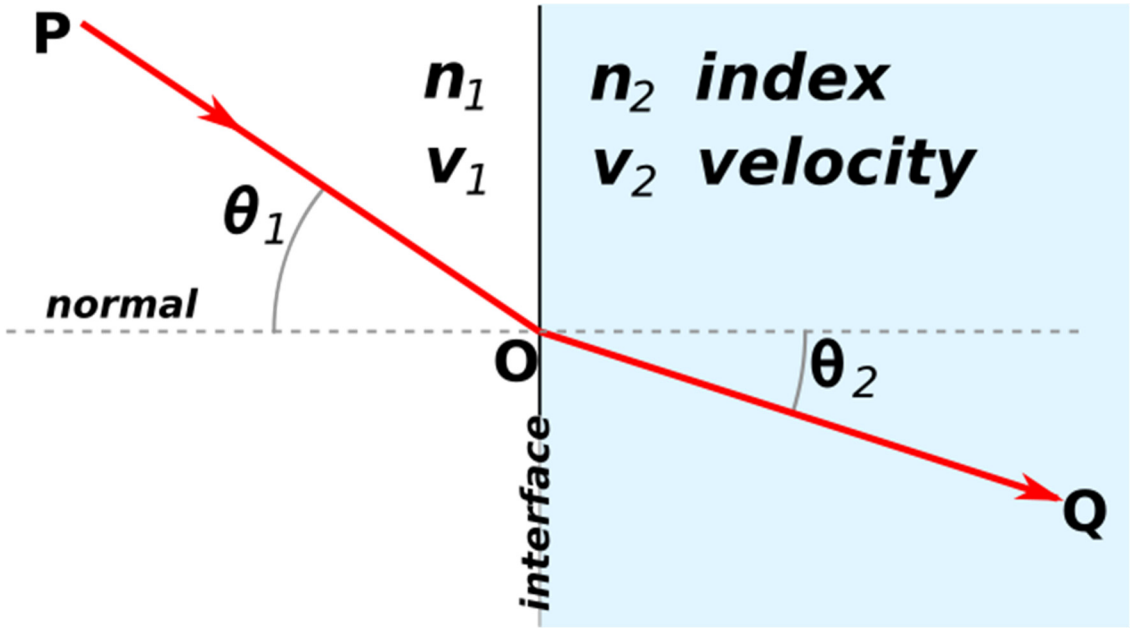
Snell's law

$$\frac{\sin\theta_1}{\sin\theta_2} = \frac{n_2}{n_1}$$

Index of refraction

$$n = \frac{c}{v_\mathrm{p}}.$$

The speed of light in a material depends on the properties of the material



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Birefringence

Birefringence, or double refraction

Icelandic Spar / Calcite / $CaCO_3$
Used by the Vikings for navigation (sunstone)

- To tell the direction of the sun on a cloudy day

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What is Birefringence?

Indexes of refraction depend on polarization

The birefringent effect (using calcite) was first described by scientist Rasmus Bartholin in 1669

If a crystal has two different Indexes of refraction: n_e and n_o

Birefringence

$$\Delta n = n_e - n_o$$

Calcite: Δn(590nm) = 1.486 – 1.64 = - 0.154

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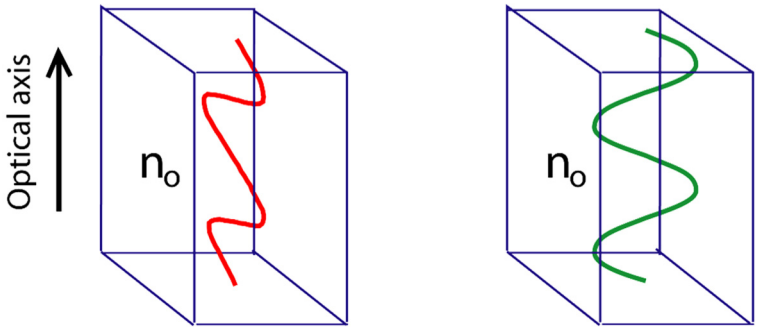
What is Birefringence

What is meant by two indexes of refraction?

The material (crystal) has
a direction: the optical axis

Light travelling along
the optical axis:

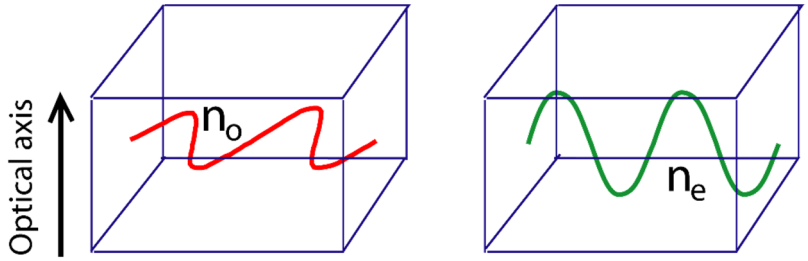
n independent of polarization



Light travelling perpendicular
to the optical axis:
n dependents on polarization

Pol. ⊥optical axis: n_o

Pol. || optical axis: n_e



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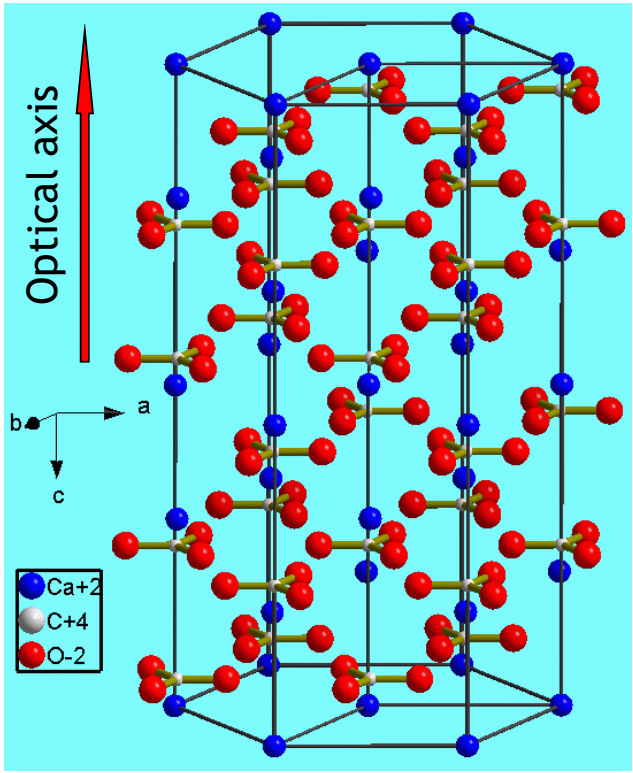
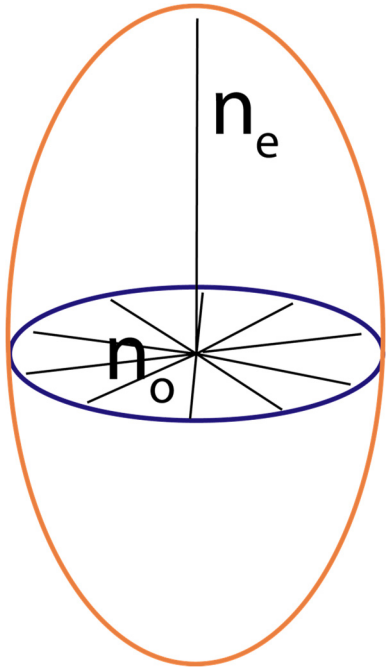
Origin of Birefringence

Crystal structure of Calcite



Calcite is an uniaxial crystal



Birefringence

$$\Delta n = n_e - n_o$$

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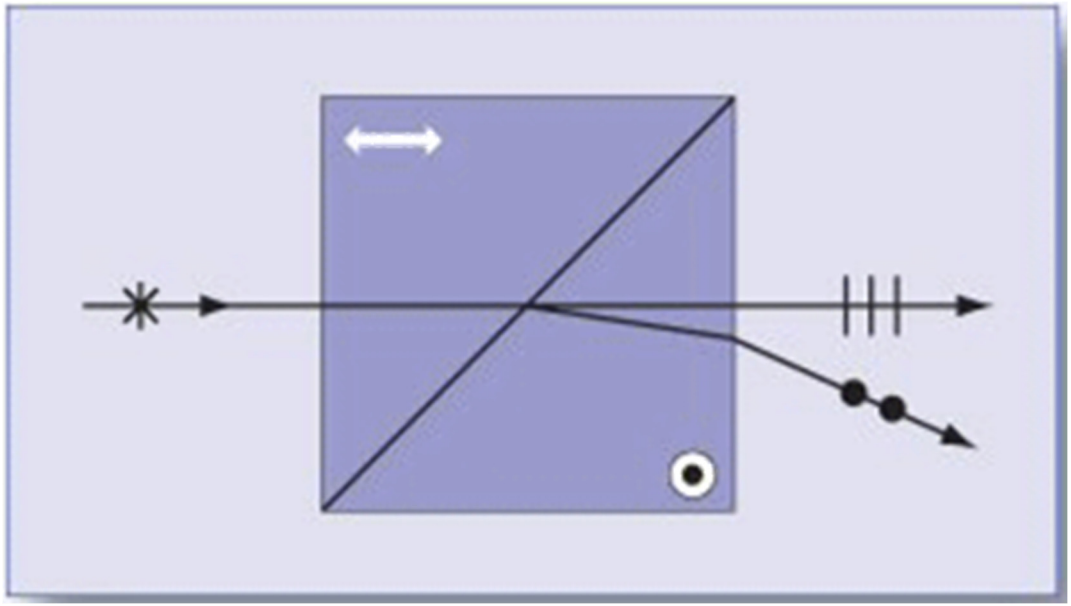
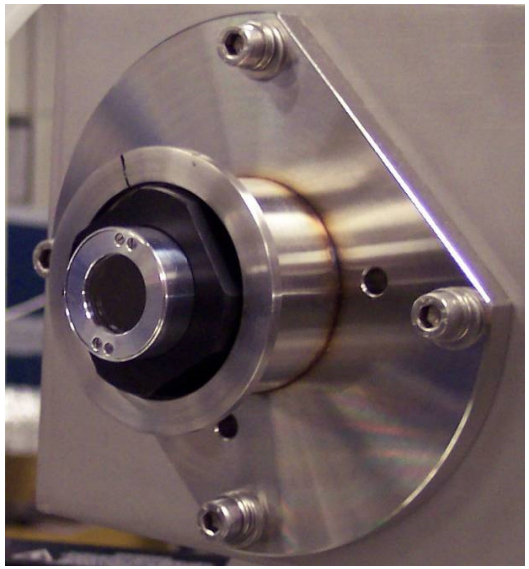
The Rochon Polarizer

"MgF2 is slightly birefringent…"

- Info on MgF_2 from Crystran Ltd., UK.

MgF_2: $\Delta n(546nm) = 1.390 - 1.379 = 0.011$ $(<< \Delta n_{Calcite})$





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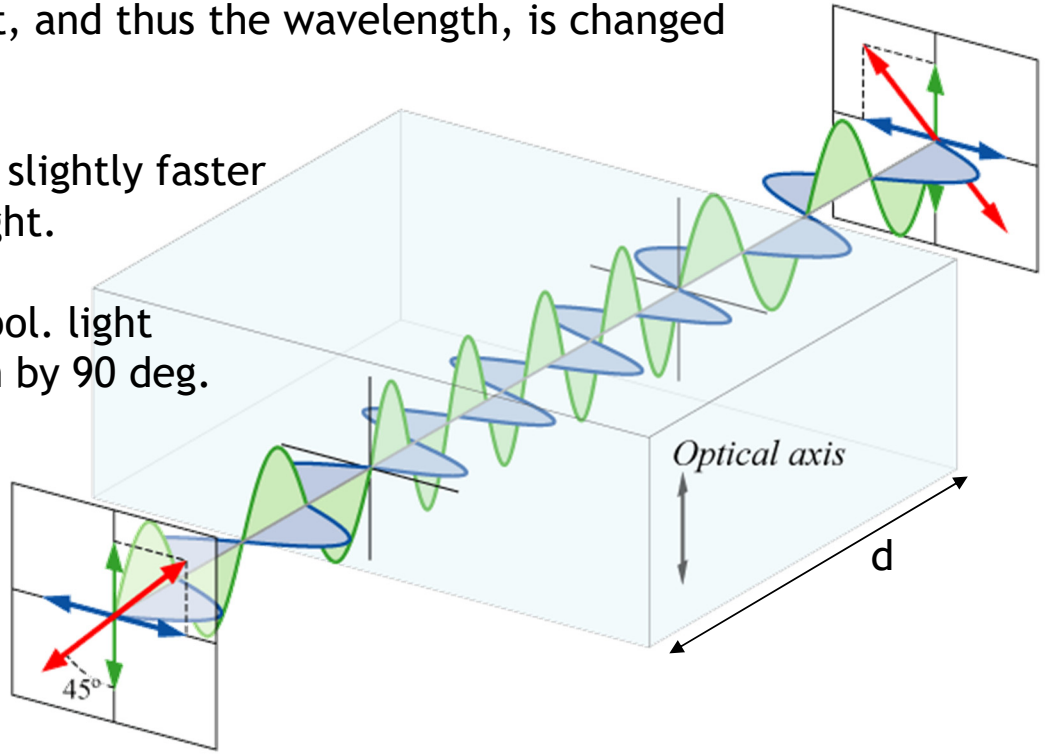
Retarders/Wave plates

- The speed of the light, and thus the wavelength, is changed inside the crystal
- The horz. pol. light is slightly faster than the vert. pol. light.
- The initially 45 deg. pol. light ‘flips’ the polarization by 90 deg.

½ waveplate



Retardation

$R = (n_e - n_o)\, d / \lambda$

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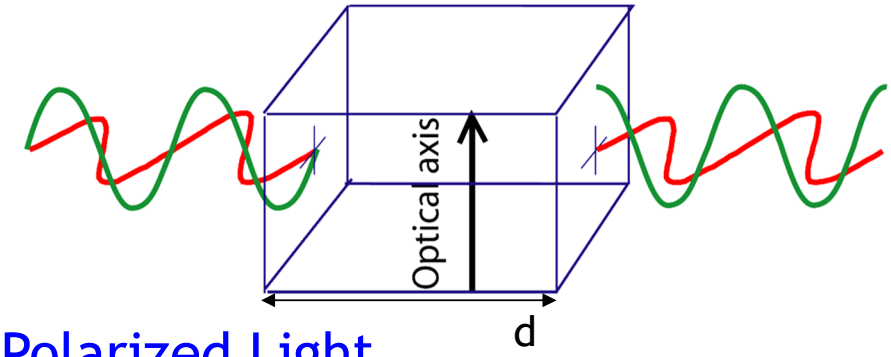
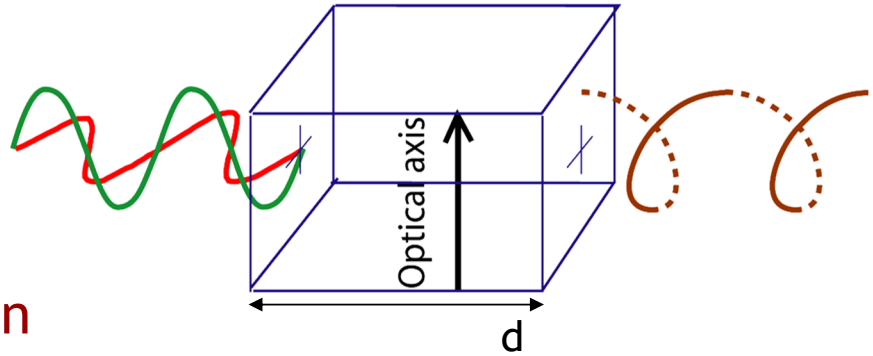
Retarders/Wave plates

Half the thickness: → ¼ waveplate



Circularly Polarized Light



Retardation

$R = (n_e - n_o)\, d / \lambda = ¼$

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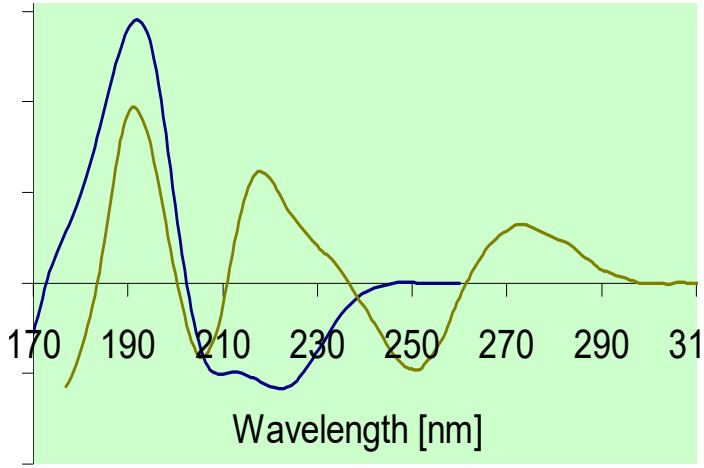
Photo Elastic Modulators (PEM)

- We are scanning the wavelength
- We can't change d as we scan



To keep R = 1/4 (circularly pol) change n_e-n_o by:

Compress ..... or ..... Stretch CaF_2

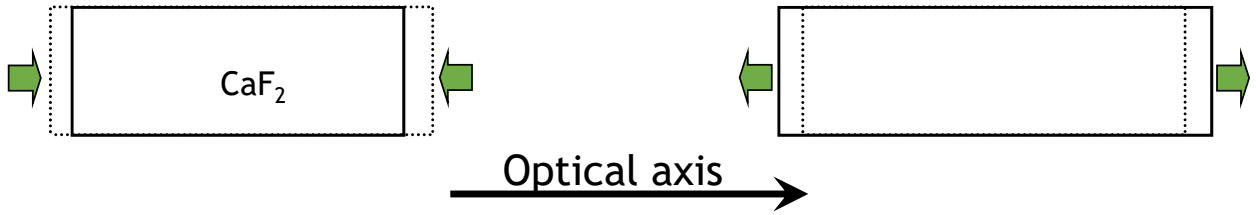


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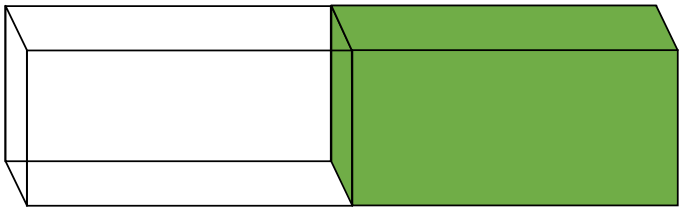
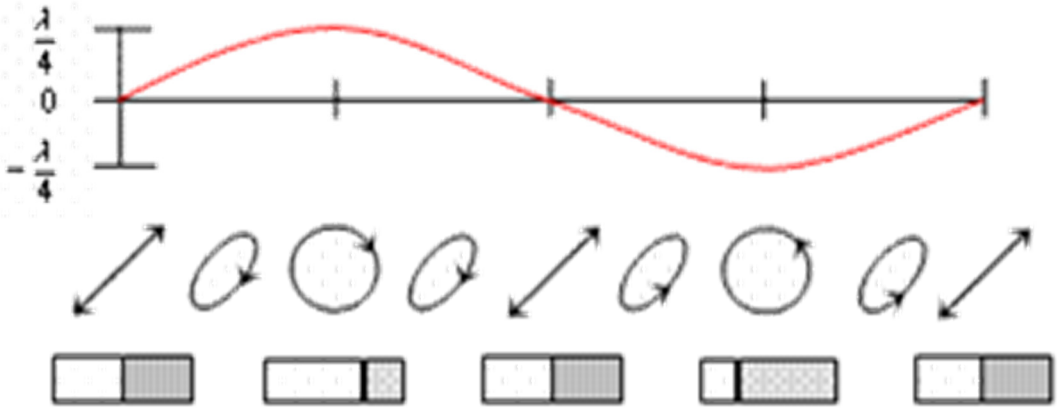
Photo Elastic Modulators (PEM)

CaF_2 crystal



Piezo crystal.

Vibrates at ~50 kHz



As the crystal vibrates the polarization is changed:

Lin. → Left Circ. → Lin. → Right Circ. → Lin. → …

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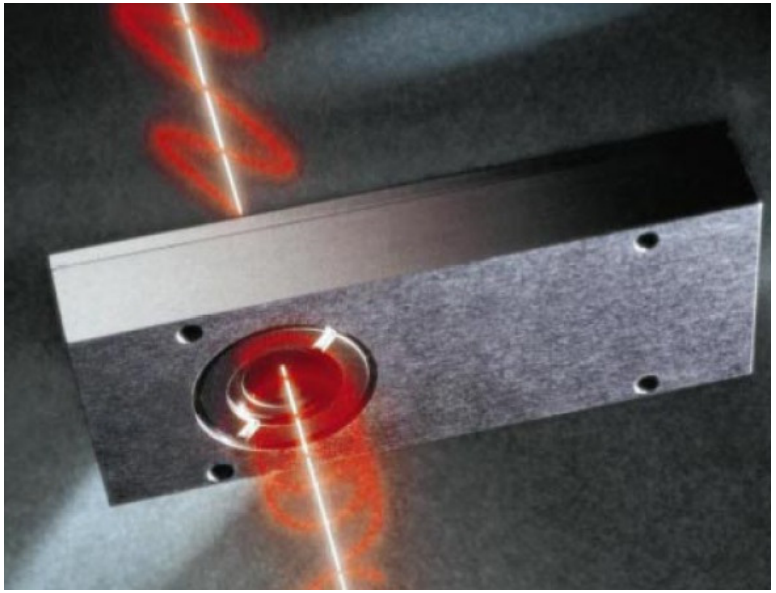
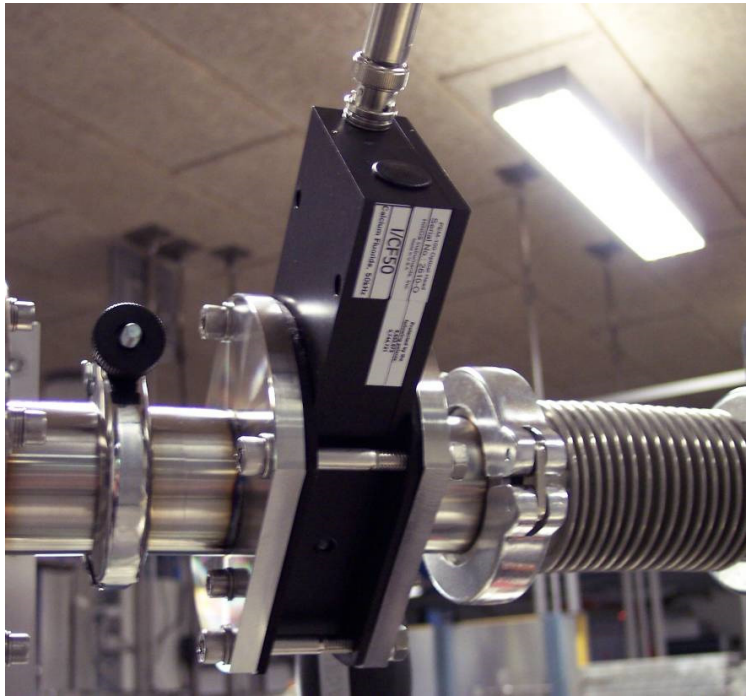
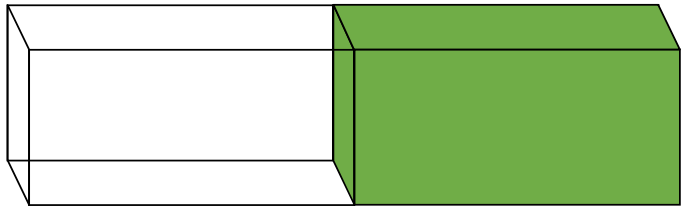
Photo Elastic Modulators (PEM)





Linear pol. is converted to Circ. pol.

CaF_2 crystal



Piezo crystal.

Vibrates at ~50 kHz

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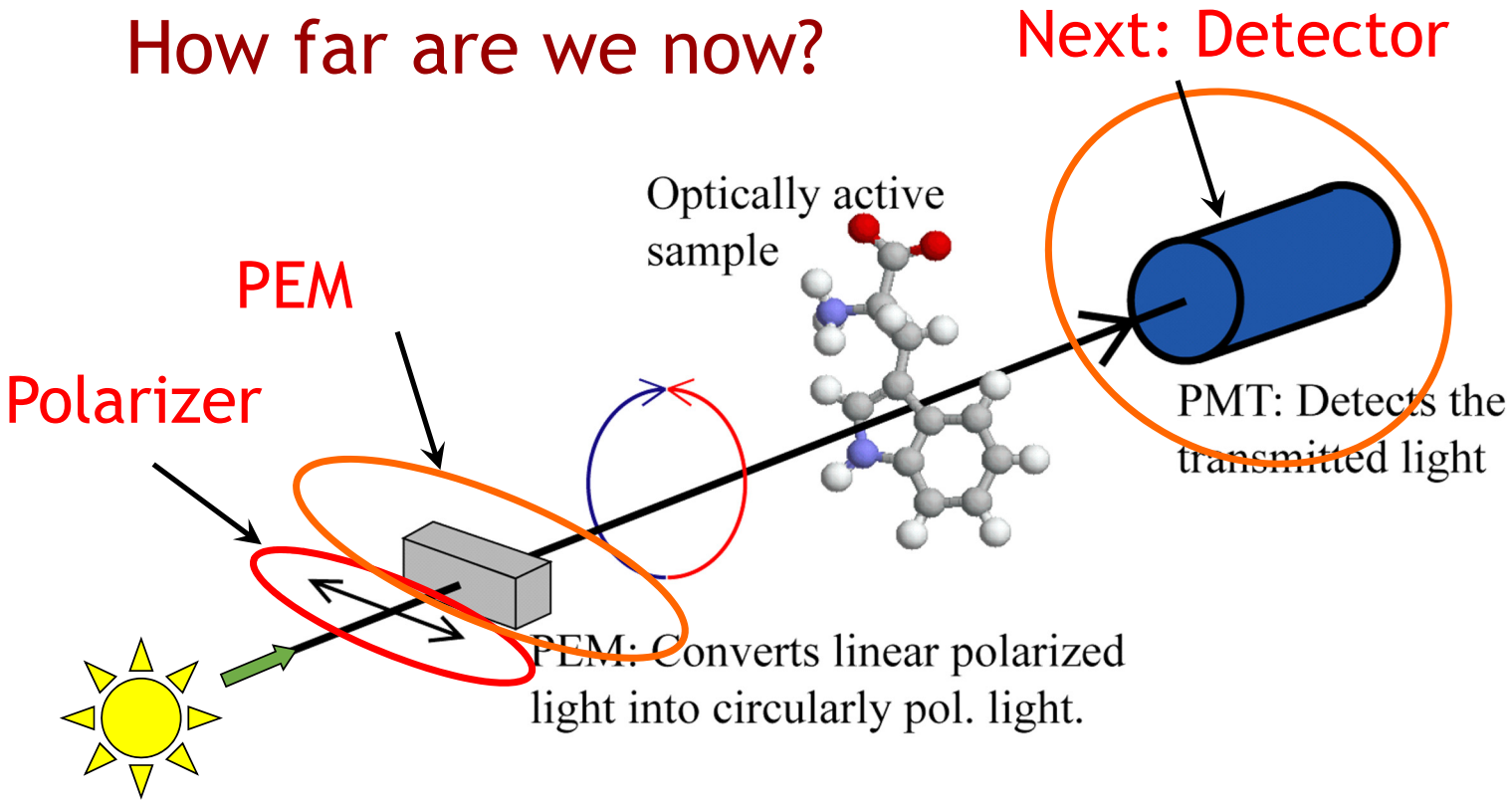
Circular Dichroism

How far are we now?



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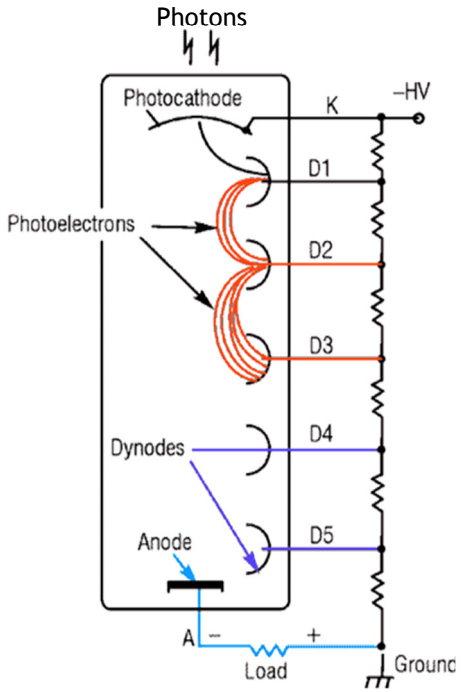
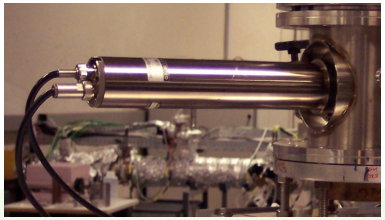
CD/UV-VIS Instrumentation

Photomultiplier Tube (PMT) Detector





- A photon is converted to an electron on the cathode:
  Q_{eff} (quantum efficiency)
- The cathode can be optimized to certain wavelength ranges
  UV, VIS or even Solar Blind
- A high voltage (HV) drop is applied along the dynodes
- The photon electron is amplified to many electrons:
  Gain(HV) (strongly depends on HV)
  $Gain(HV) \approx b \cdot (HV)^a$
- The electrons are collected on the Anode:
  Either pulses or a current is detected

Typically: $Gain(HV) \approx 10^3 - 10^7$ and $Q_{eff} < 1$

$$I_{Anode}(\lambda, HV) = Flux\ [ph/sec] \cdot e \cdot Q_{eff}(\lambda) \cdot Gain(HV)$$

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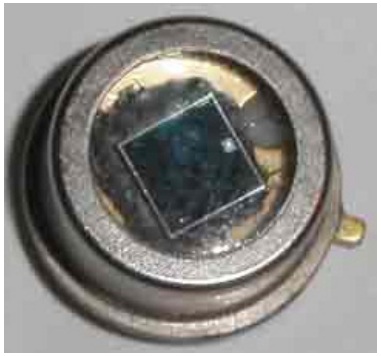
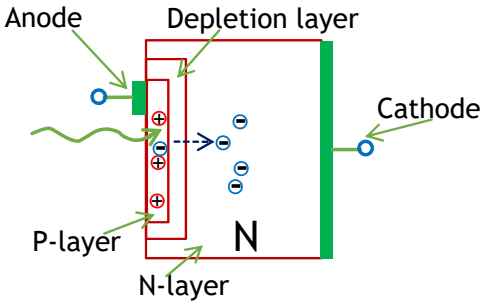
CD/UV-VIS Instrumentation

Photodiode





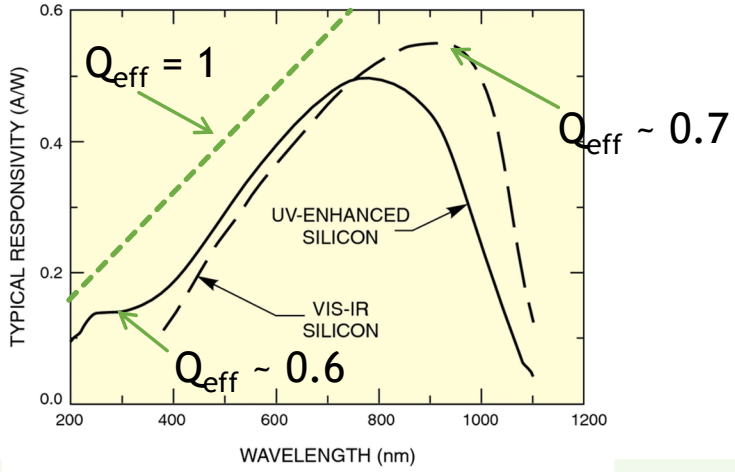
- The diode converts photons to a current
- Q_{eff} can be as high as 80%
- Spectral range depends on material
  E.g. Si 190 - 1100 nm, Ge 400 – 1700 nm

Advantage:

Very rugged, high Q_{eff} (in the VIS/IR)

Disadvantage:

No gain , although avalanche diodes can have a gain up to 1000



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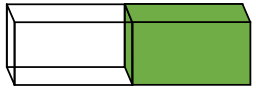
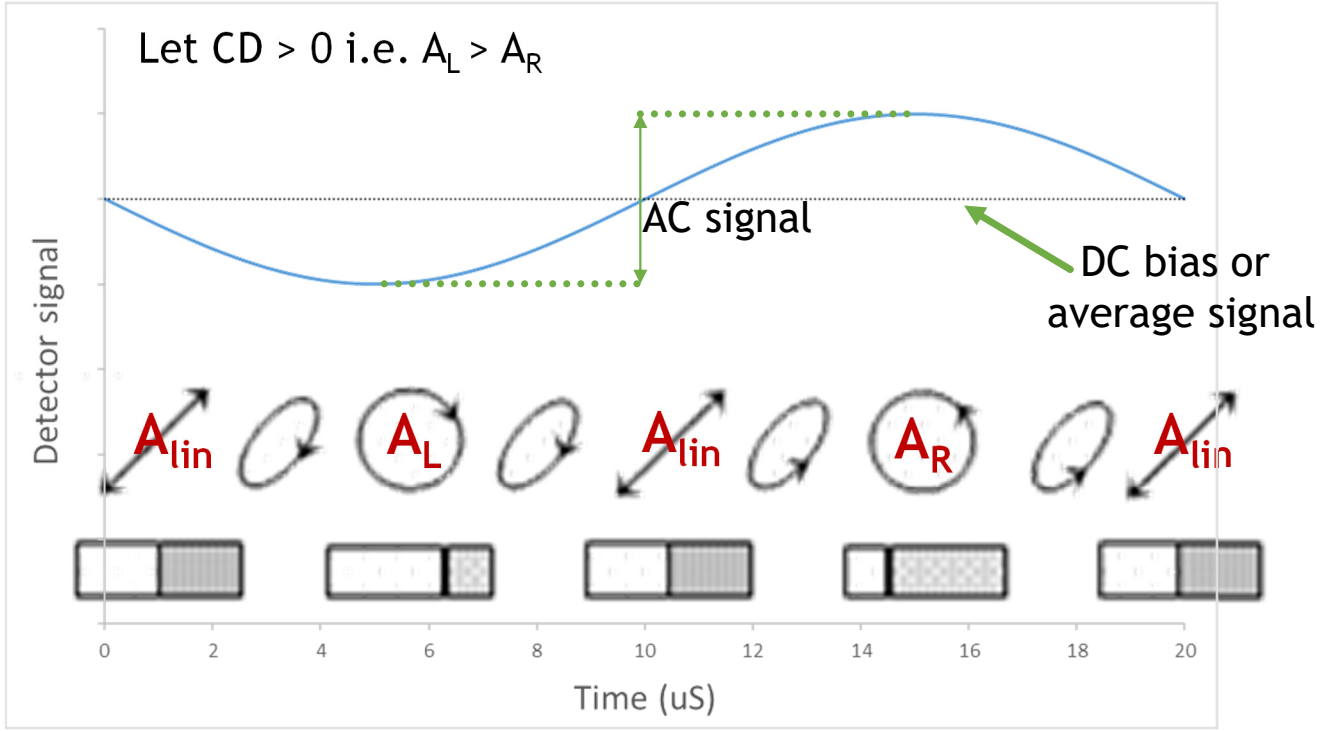
The signal from the detector

The PEM oscillates and changes the polarization  Vibrates at ~50 kHz

- Polarization L ⟹ absorbance A_L
- Polarization R ⟹ absorbance A_R

• Polarization is linear ⟹ $A_{lin} = ½(A_L + A_R)$



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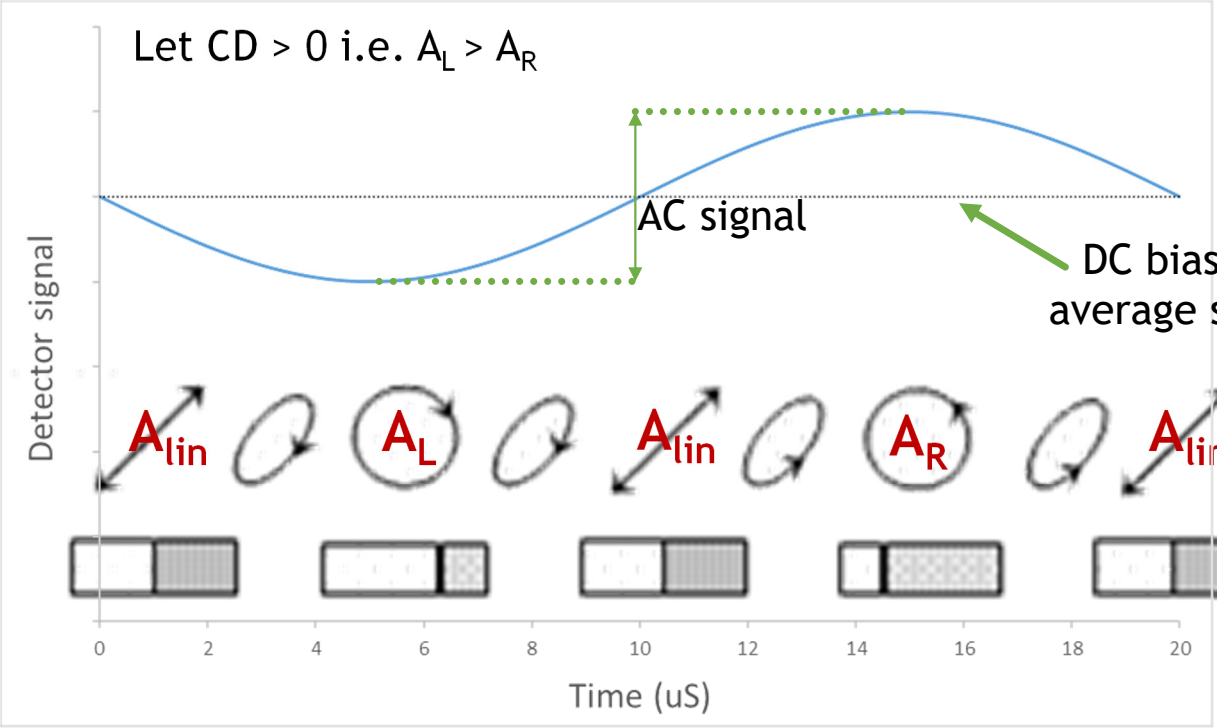
The signal from the detector



The CD instrument measures the *AC* (and *DC*)

$$CD = constant \times \frac{AC}{DC}$$

*In most instruments the DC is kept constant by **changing the detector high voltage (HT)***

$$CD = const \times AC$$

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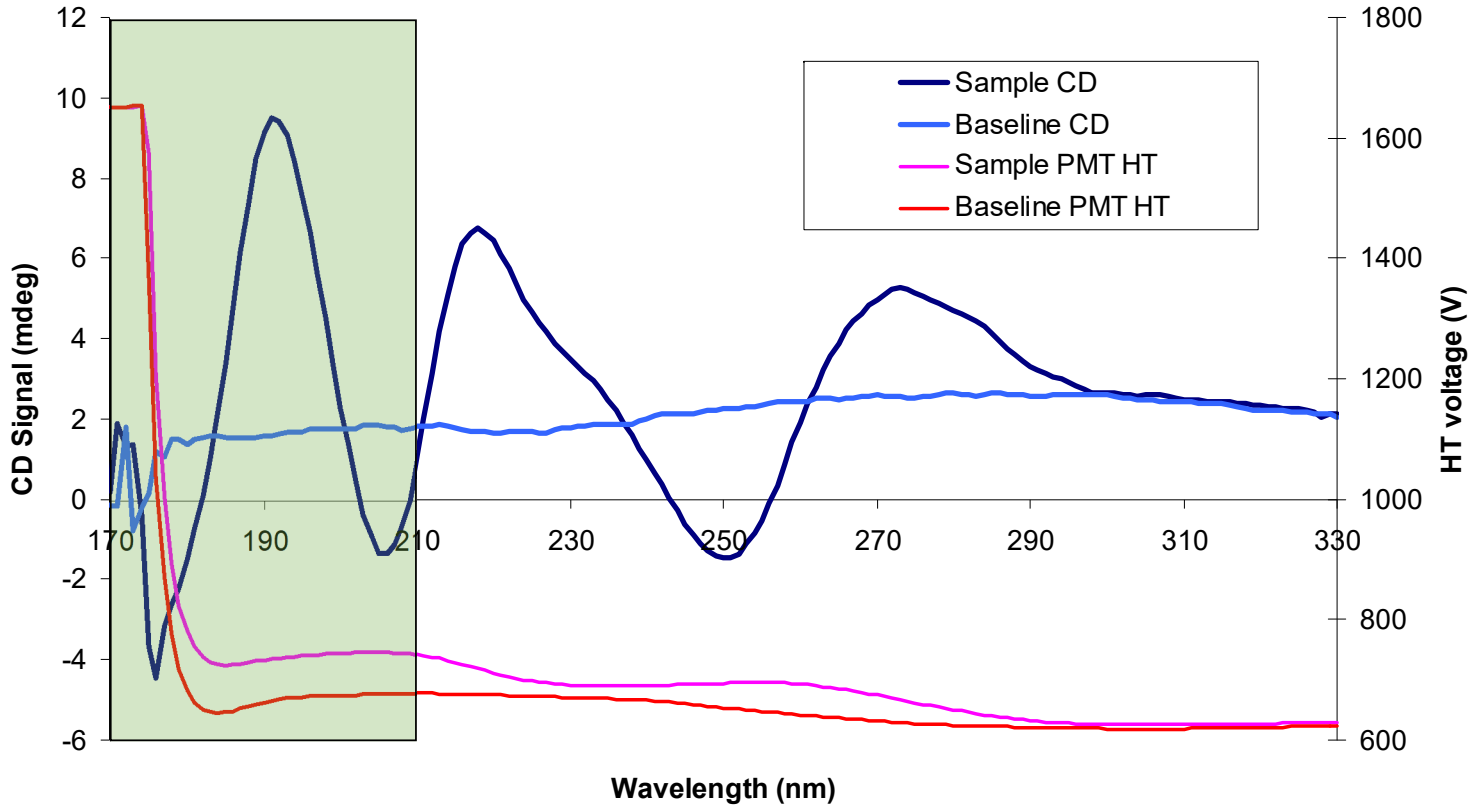
What does the HT tell us?

High HT ⟺ Low photon flux reaching detector ⟺ High Absorbance



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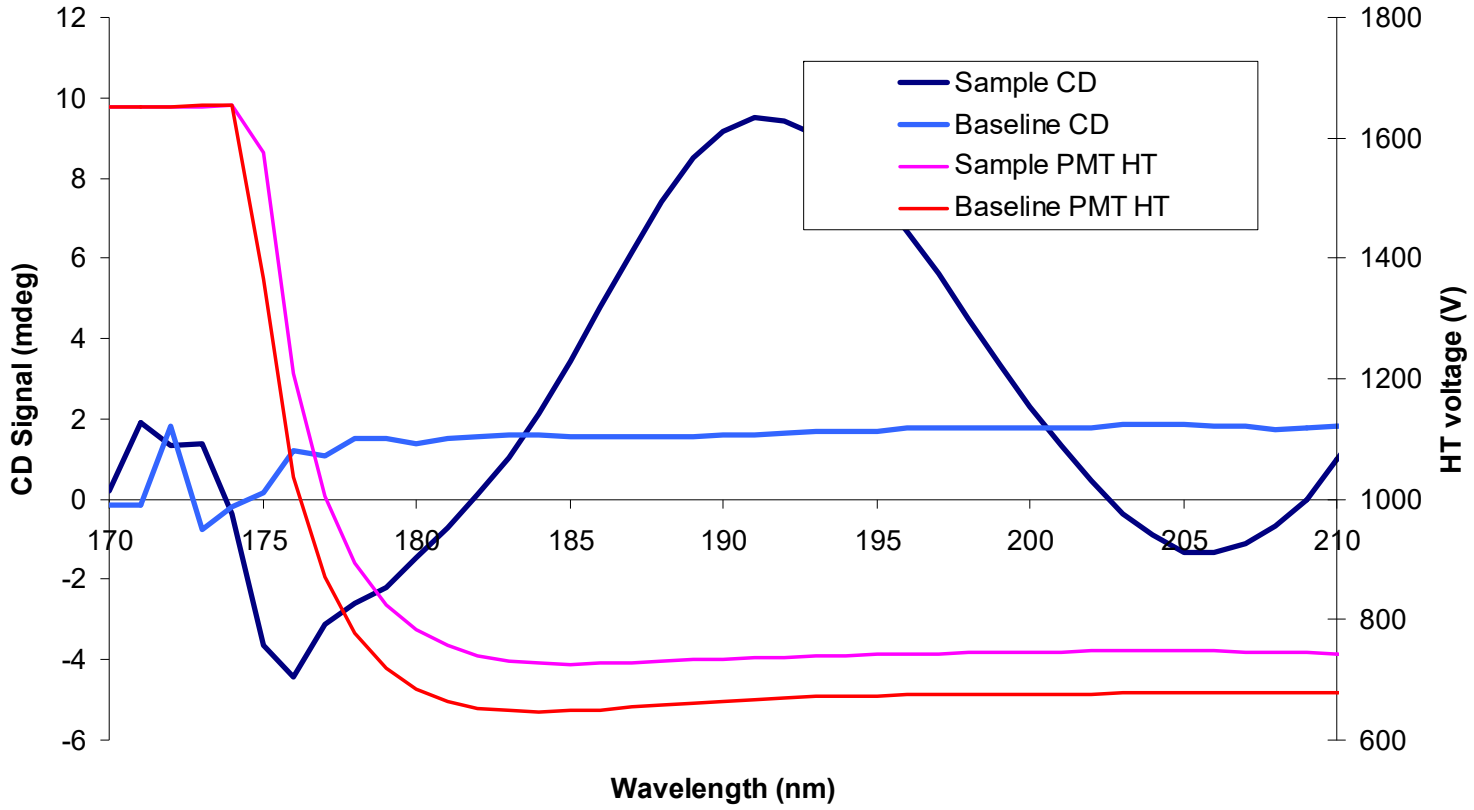

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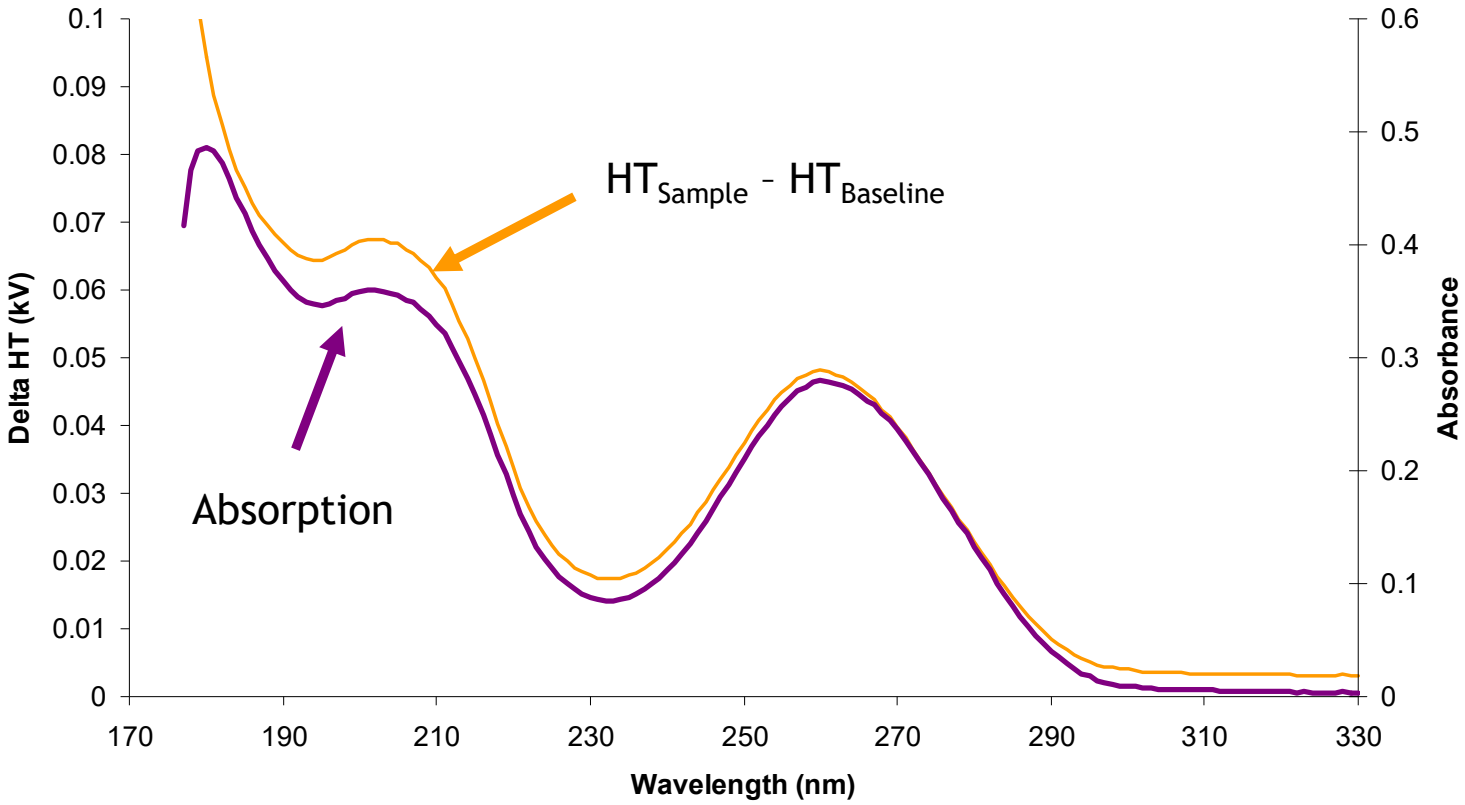
What does the HT tell us?

Delta HT (HT_{Sample} – $HT_{Baseline}$) is a 'pseudo' absorption



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What does the HT tell us?

It is possible to use HT_{Sample} and $HT_{Baseline}$ to calculate the absorbance

- Record the HT_{Sample} (λ) and $HT_{Baseline}$ (λ) together with the CD signal
- The average signal from the detector is constant (DC bias)
- Assume that for sample and baseline these are the same:
  - *Lamp output*
  - *Optics transmission*
- Assume the gain of the detector vs. HT is $Gain(HT) = b \times HT^a$

$$Detector\ signal_{sample} = Detector\ signal_{baseline}$$

$$10^{-A_{sample}(\lambda)} \times Gain(HT_{sample}(\lambda)) = 10^{-A_{baseline}(\lambda)} \times Gain(HT_{baseline}(\lambda))$$

Use $Gain(HT) = b \times HT^a$: $$10^{A_{sample}(\lambda) - A_{baseline}(\lambda)} = \frac{b \times (HT_{sample}(\lambda))^a}{b \times (HT_{baseline}(\lambda))^a}$$

$$A(\lambda) = a \times \log\left(\frac{HT_{sample}(\lambda)}{HT_{baseline}(\lambda)}\right)$$

Only depends on a **single constant *a*** !
Calibrate this using samples with know concentrations

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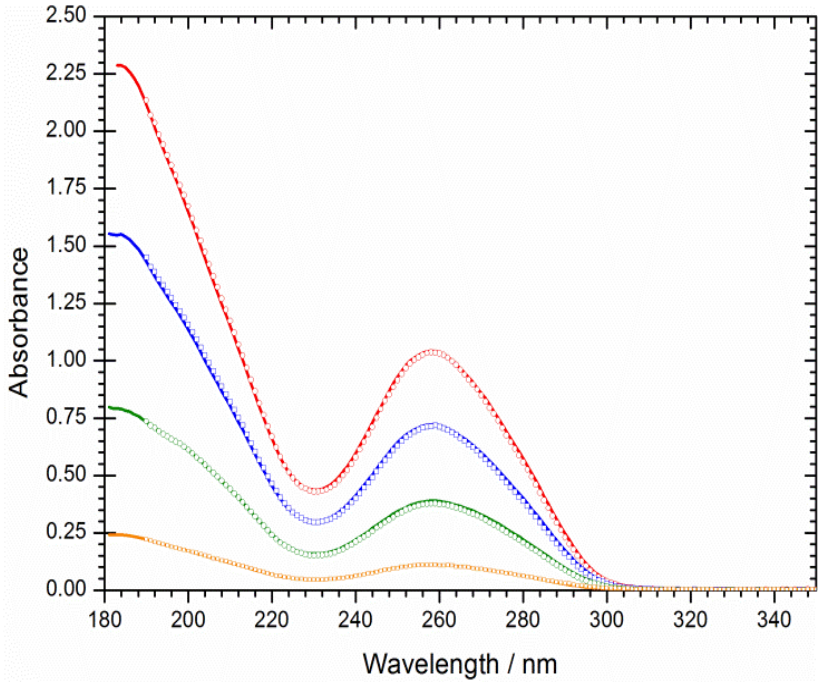
What does the HT tell us?

*Highly accurate **simultaneous** CD and Absorption measurements*

$$A(\lambda) = a \times \log\left(\frac{HT_{sample}(\lambda)}{HT_{baseline}(\lambda)}\right)$$

Full range of absorptions 0-2 calibrated with a **single** detector parameter



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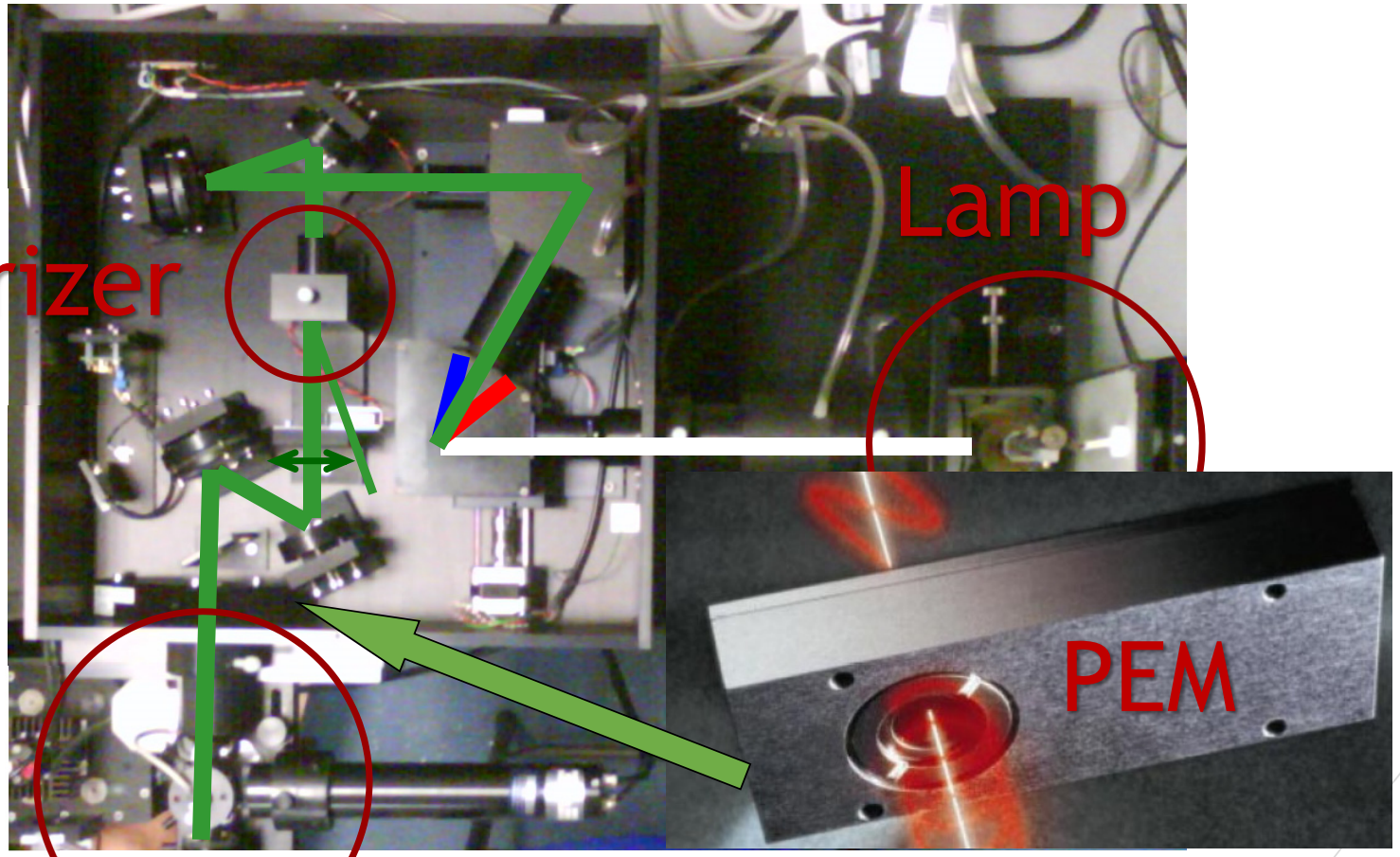
Instrumentation

Conventional CD instrument (lamp based)



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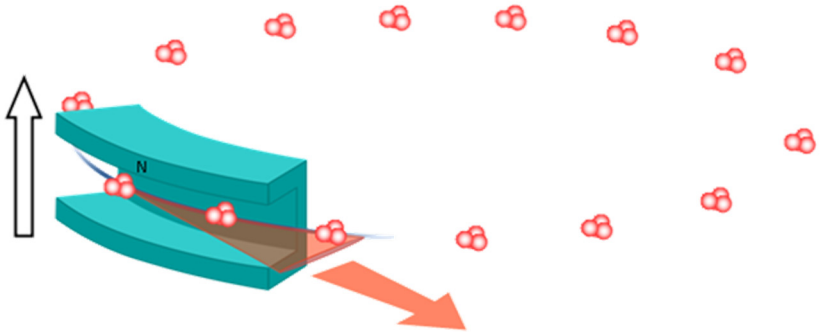
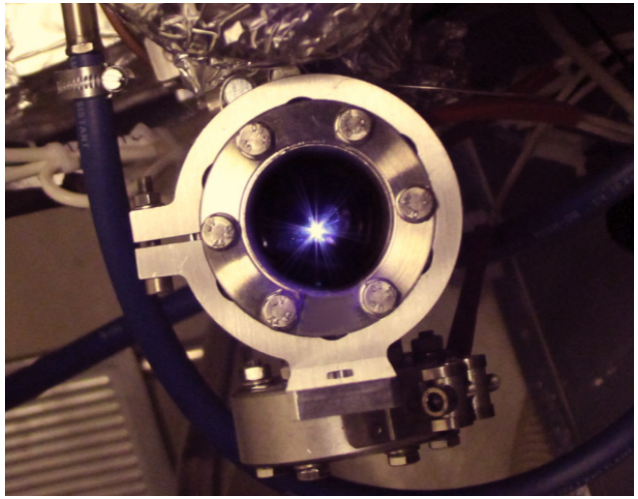
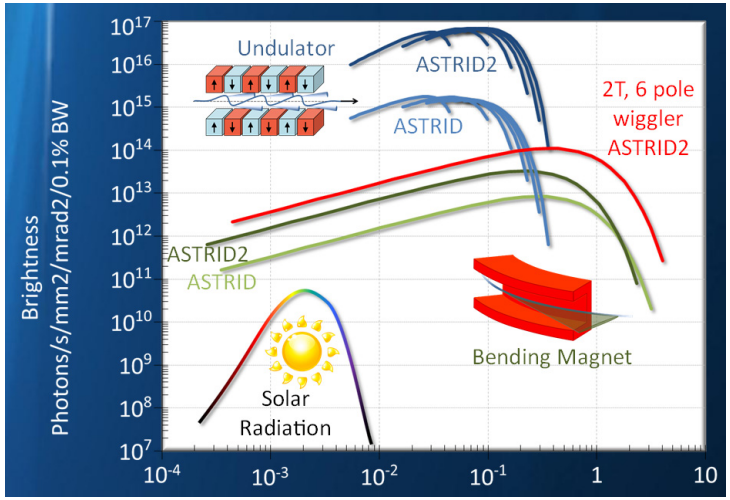
Other options for light sources: Synchrotron Radiation (SR)

- UV light is well suited to examine molecules like proteins and DNA.
- Synchrotron radiation (SR) is emitted when charged particles are accelerated: We use electrons at relativistic speeds.
- The light is VERY intense.







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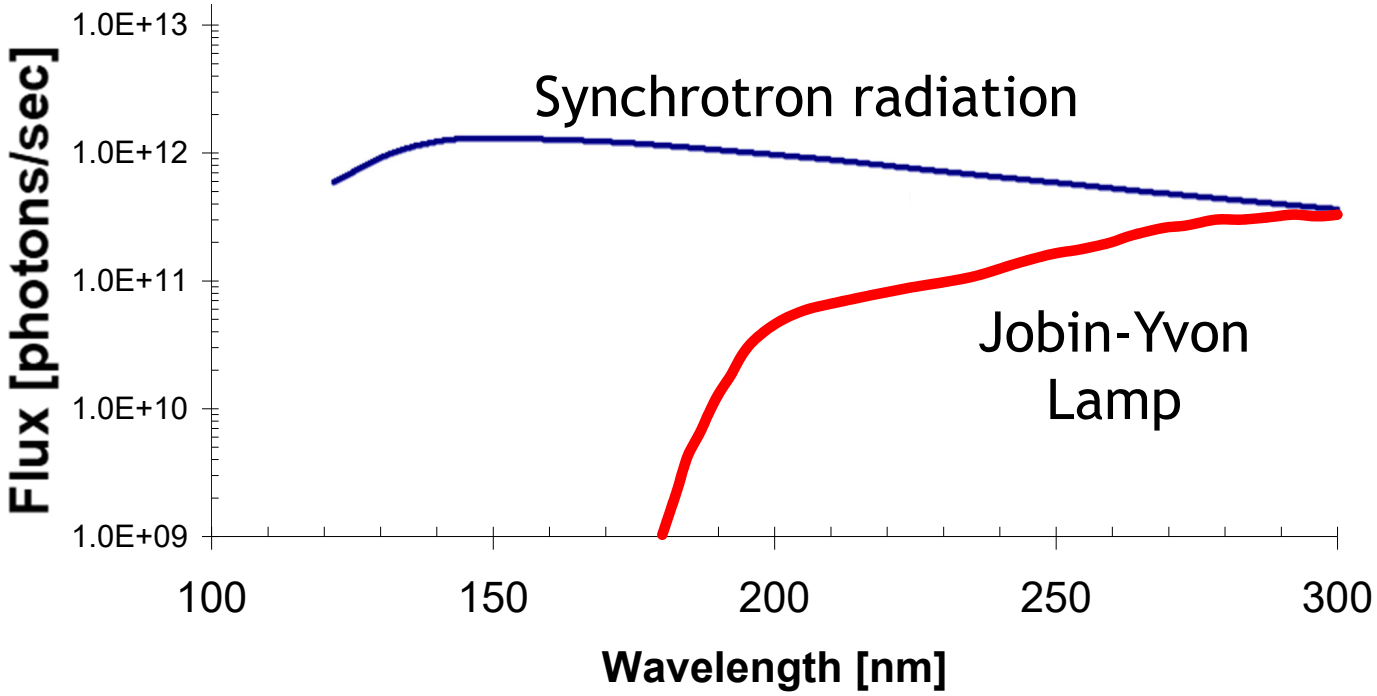
Other options for light sources: SR vs lamps



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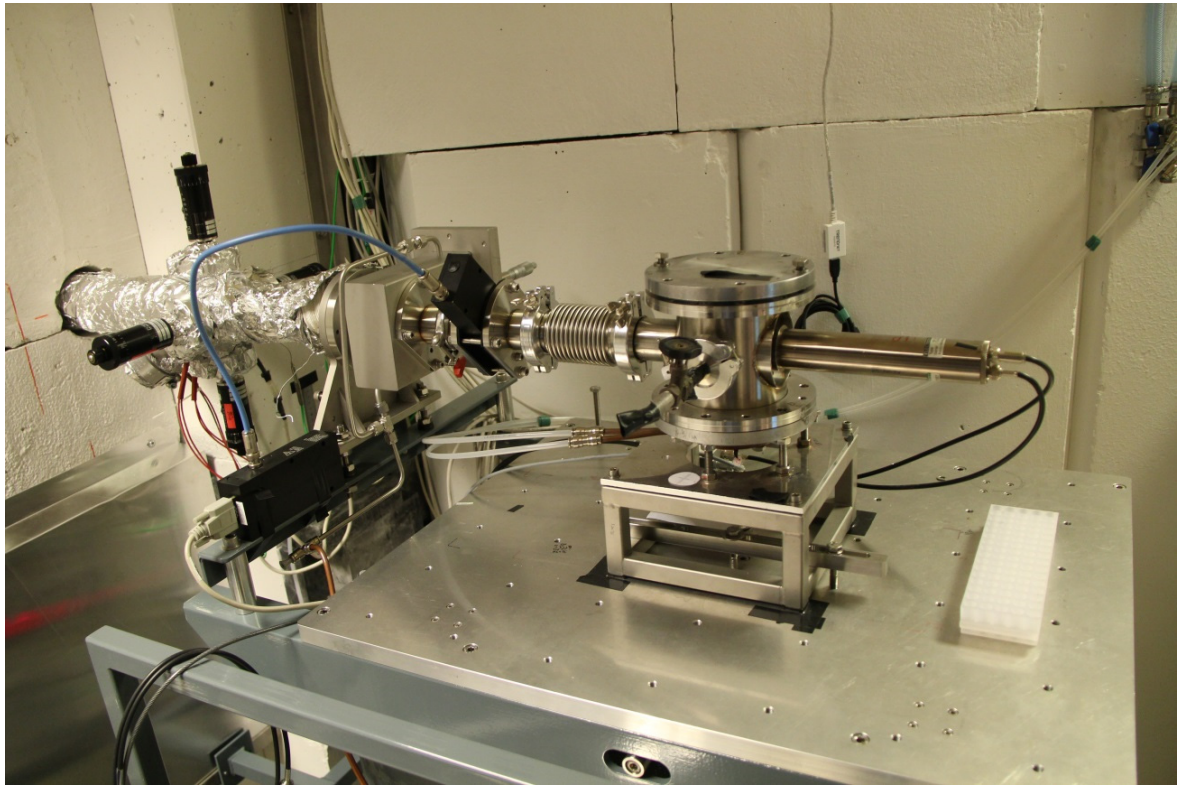
AU-CD beam line on ASTRID2

Small and compact set-up



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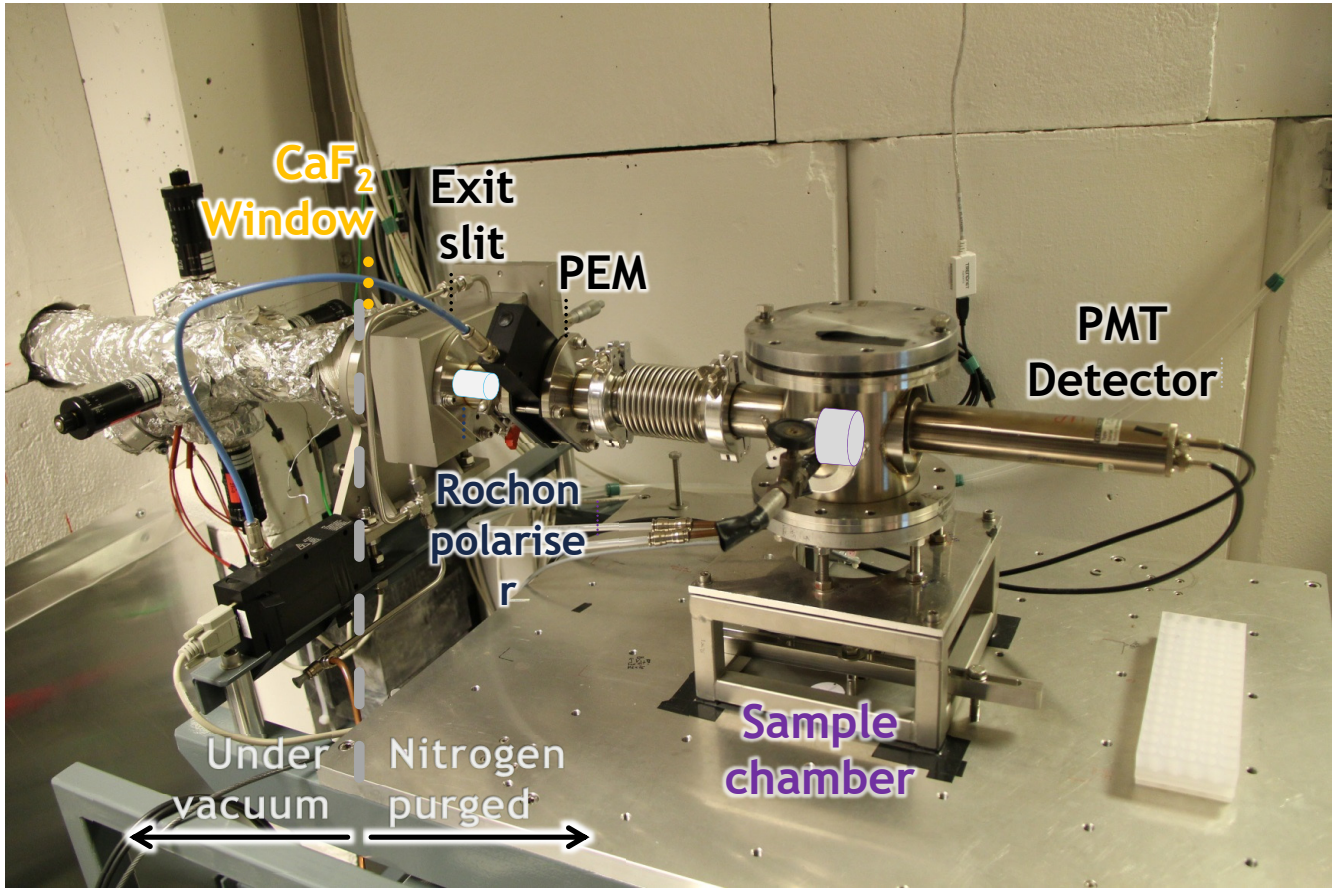
AU-CD beam line on ASTRID2



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Measurement options…

Sample holders - solvents

Cells made from Quartz suprasil with a cut-off of ~160 nm

1 cm

1 mm

- Closed cells
  - Pathlengths 0.1 mm to several cm
  - Temperature melts
- Open cells
  - Shorter pathlengths (~0.01 mm or lower!)

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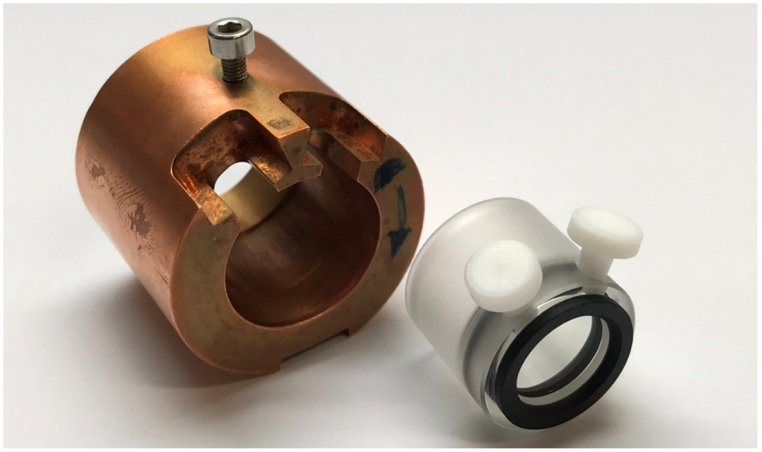
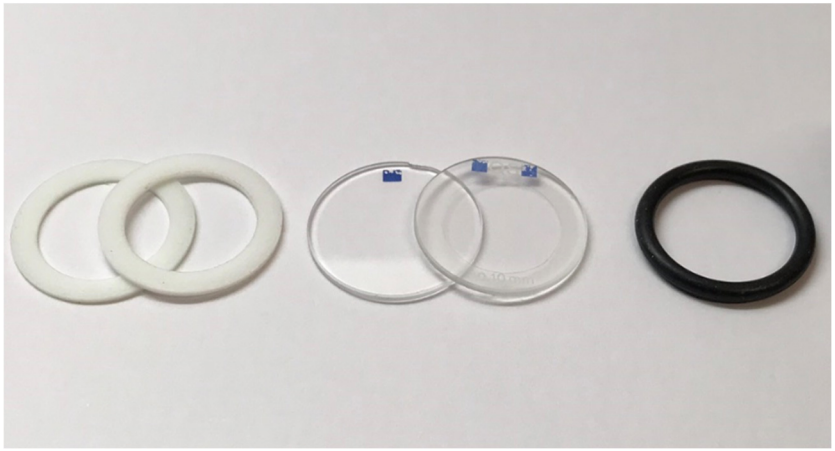
Measurement options…

Sample holders - solvents

Cells made from Quartz suprasil with a cut-off of ~160 nm

We prefer to use round cells as they often have less stress and thus birefringence





- Closed cells
  - Pathlengths 0.1 mm to several cm
  - Temperature melts
- Open cells
  - Shorter pathlengths (~0.01 mm or lower!)
  - CaF_2 cells, lower wavelength cut-off

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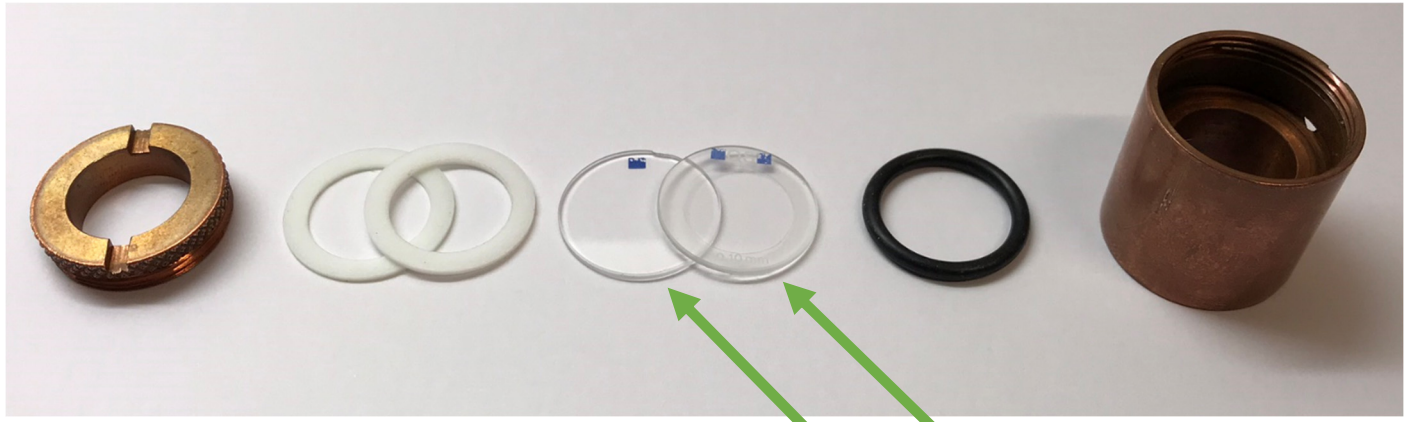

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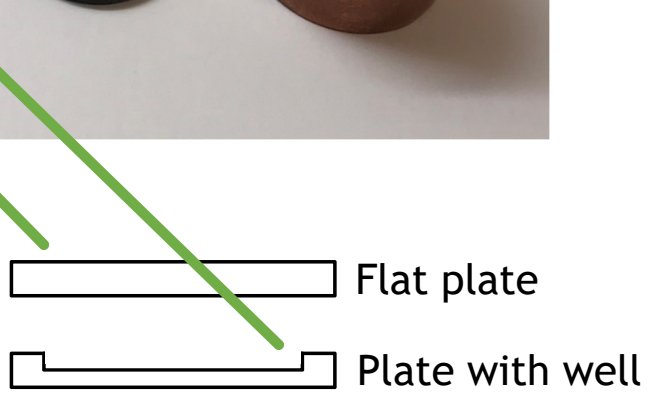

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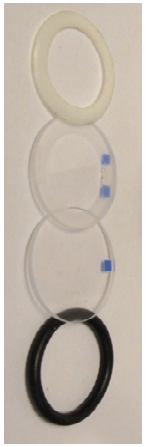
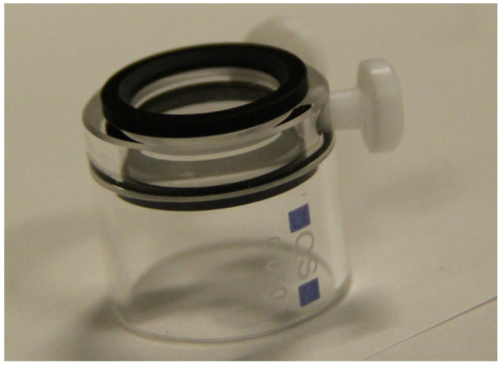
A note on cell cleaning

Some cells are easier to clean than others





Large open cuvette:

Not so difficult to clean

Use:

Water

Open cells:

Easy to clean

Use:

Water and tissue

Closed cells:

Difficult to clean

Use:

Hellmanex 2% or 10% solution and heat

Much more about this in the first Hands-on session

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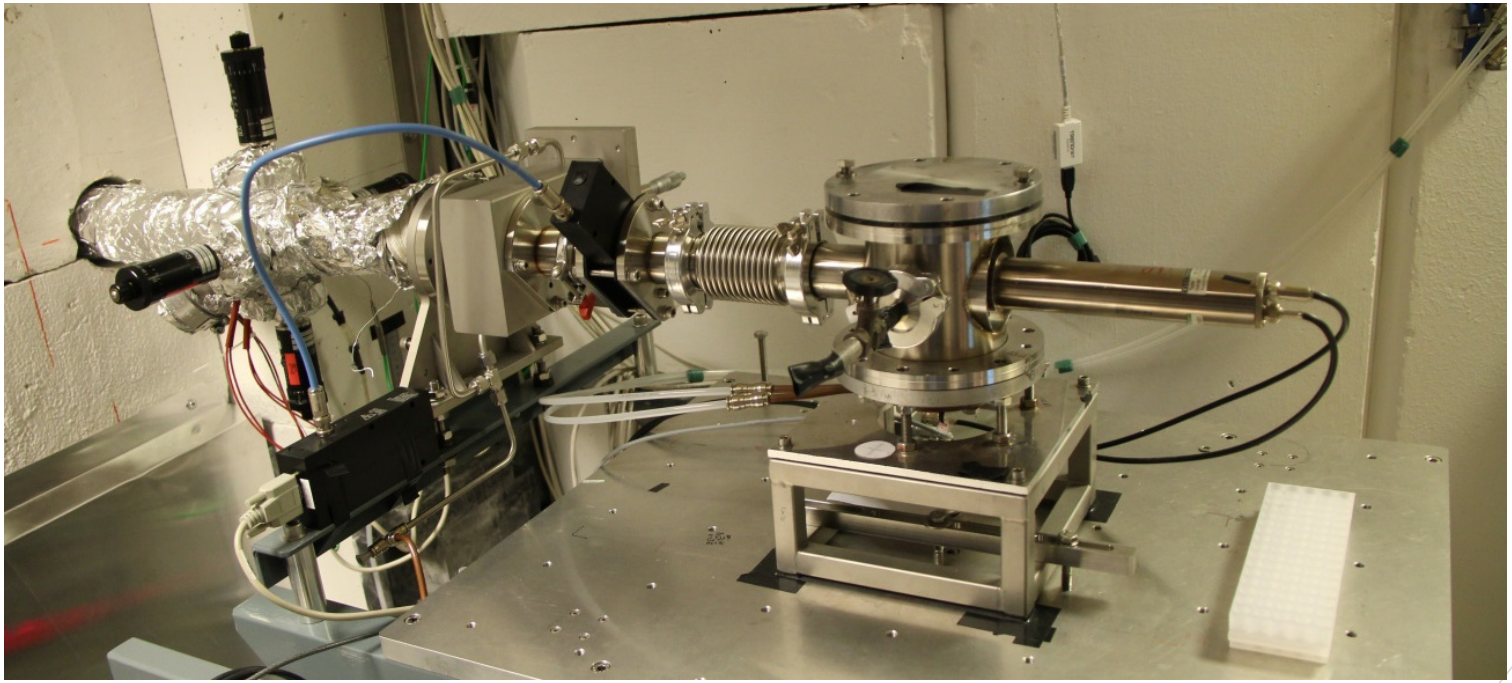
Measurement options…

- Temperature scans 5 to 90C – fully automated and integrated into the scanning programme using a macro file.



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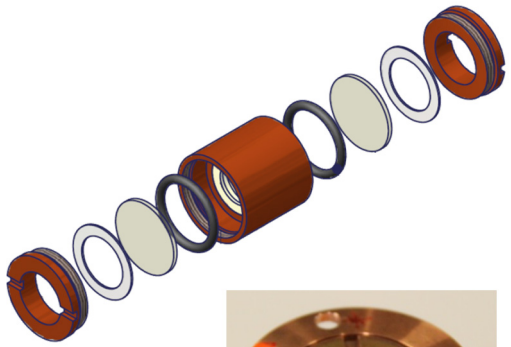
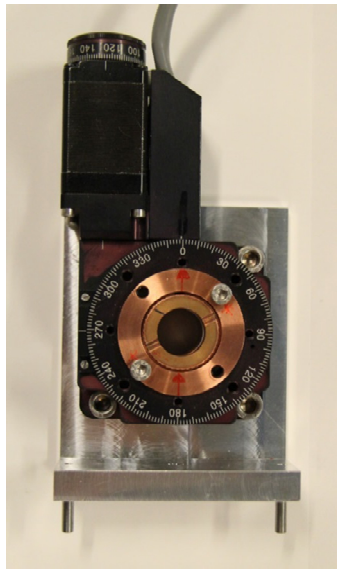
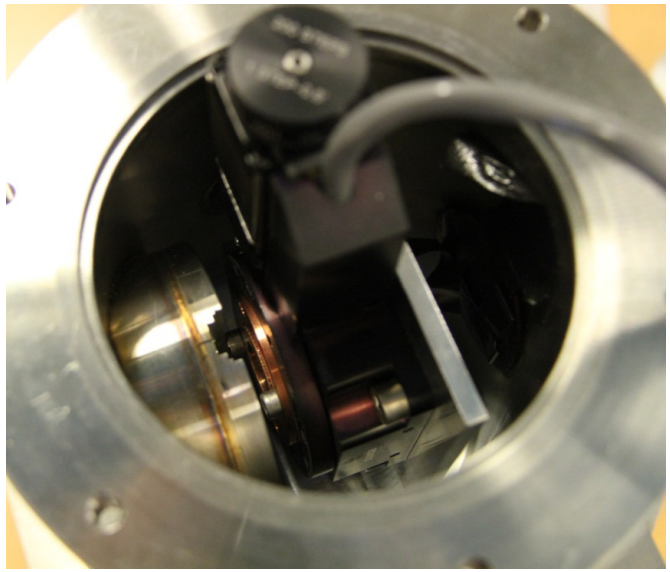
Measurement options…

- Temperature scans 5 to 90C – fully automated and integrated into the scanning programme using a macro file.
- Rotational stage







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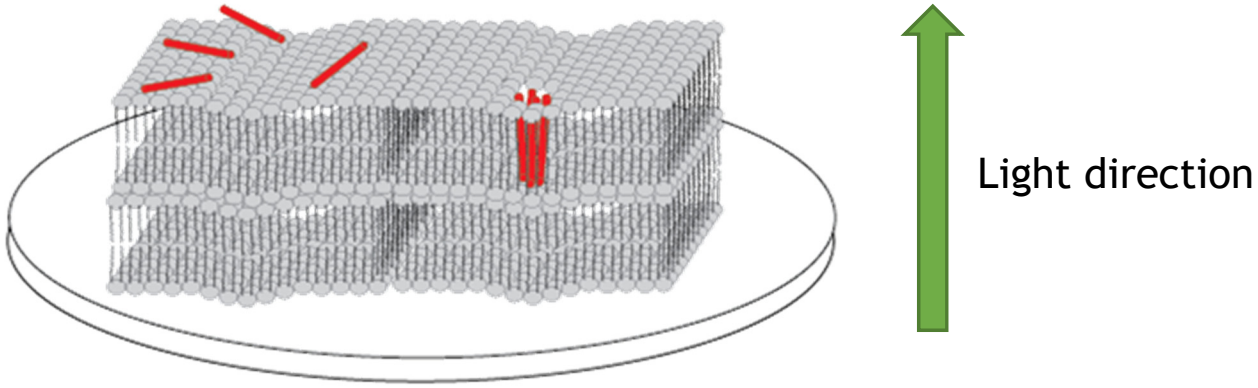
Why a rotation sample holder?

- Often used when studying films (solids) of samples
- A good example is insertion of peptides into lipid bilayers



Lipid bilayers (mixed with peptides) can be made on e.g. a quartz plate by drying a solution onto the plate.

Are the peptides inserted into the membrane or are they surface bond ?

This is a very different sample than a solution where the peptides are found in all directions with respect to the light

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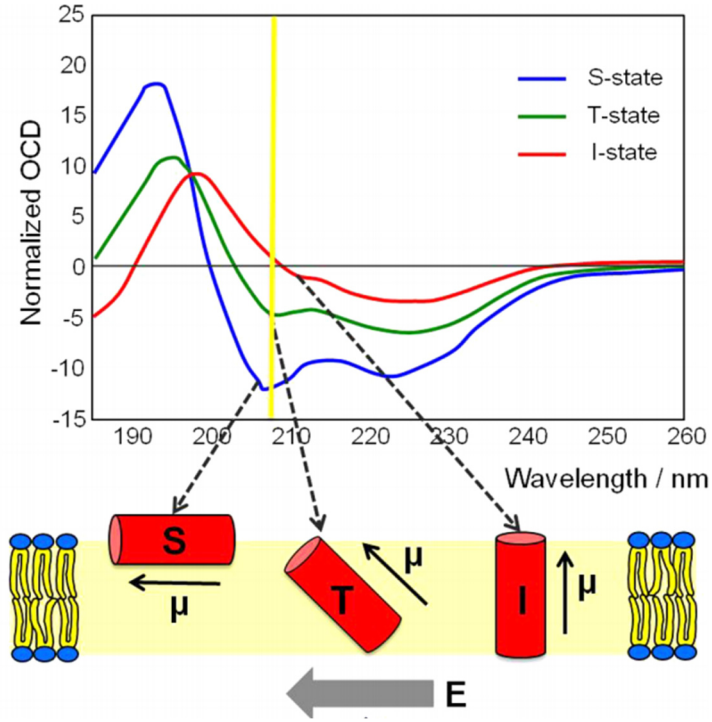
Why a rotation sample holder?

Oriented Circular Dichroism (OCD)

The CD spectrum of an helical peptide in the lipid bilayer depends on the orientation of the peptide.



S: Surface bond peptide

T: Tilted peptide

I: Inserted peptide

The 208 nm transition (**μ**) is only active when parallel to the light electric field, **E** (**μ** || **E**)

E is always perpendicular to the direction of the light

Light direction

J. Bürck *et al. Acc. Chem. Res.* 2016, 49, 184−192

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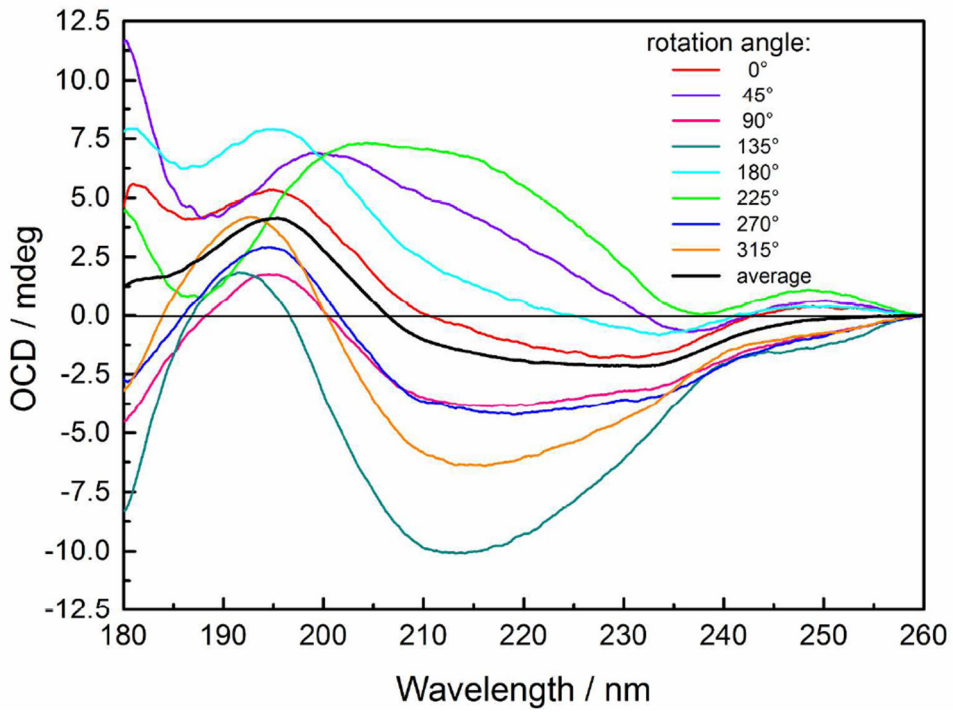
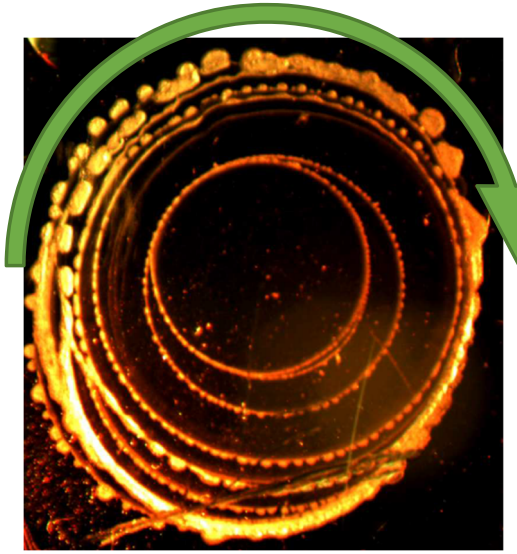
Why a rotation sample holder?

Oriented Circular Dichroism (OCD)

Such a (solid) sample is not necessarily very uniform, and may be a bit isotropic

- Shows up as changes in the CD spectrum with rotation angle





Rotation angle

A not particularly uniform sample!

The CD signal depends on sample angle, but averages to the correct spectrum!

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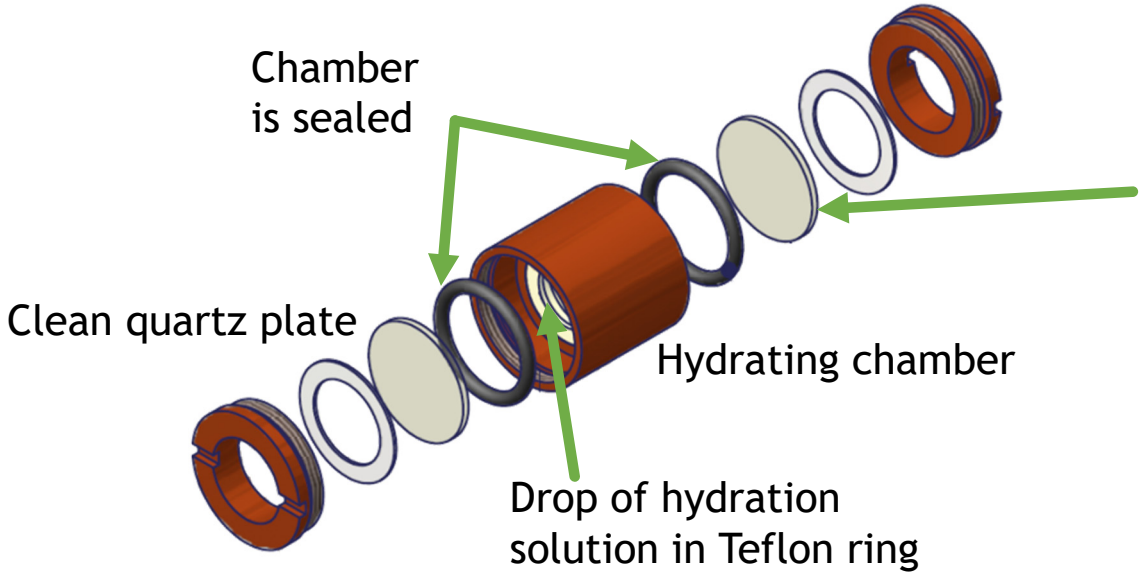
Why a rotation sample holder?

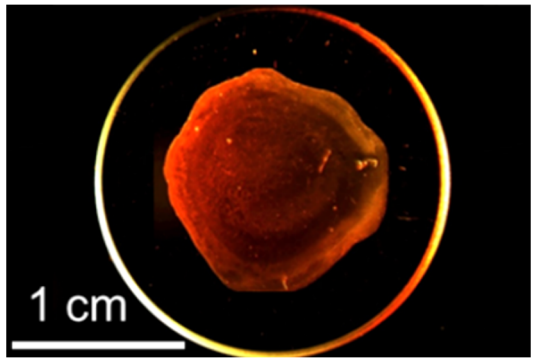
Oriented Circular Dichroism (OCD)

One important aspect of peptides in a bilayer is that they need to be kept humid and might also need to be hydrated after preparation for the peptides to insert into the membrane.

Special sample holder



Bilayers with peptide



The humidity can be controlled with different salt solutions: K_2SO_4 R.H. ~98 % $MgCl_2$ R.H. ~ 33 %

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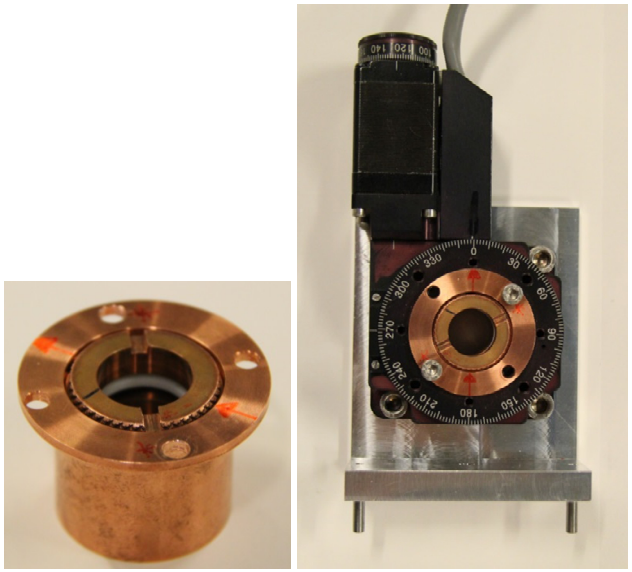
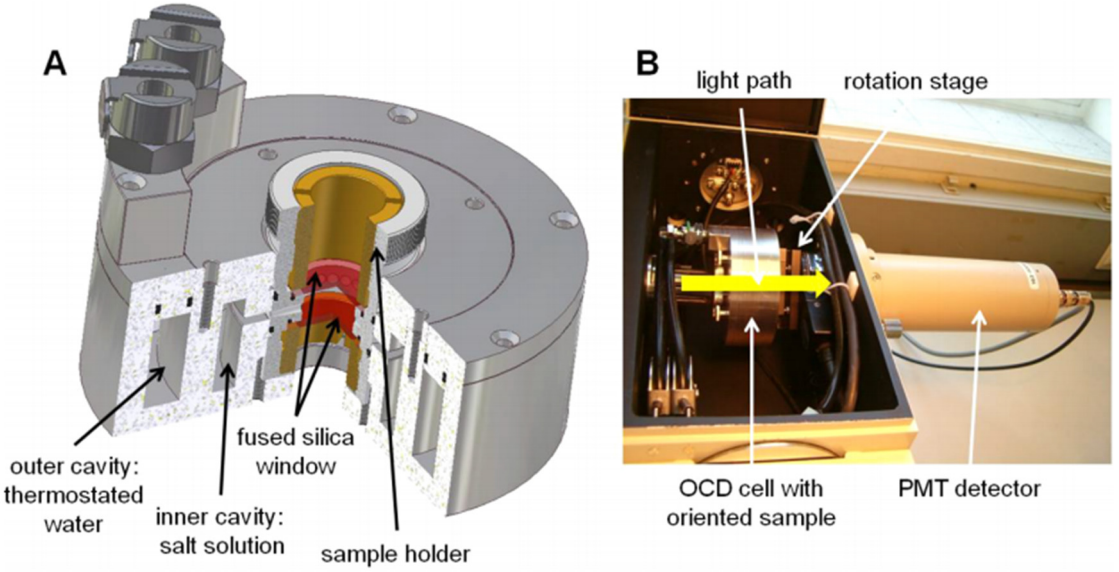
Why a rotation sample holder?

Oriented Circular Dichroism (OCD)

Our setup for rotation



KIT (DE) setup for a JASCO CD instrument



The hydrating sample holder is mounted in the rotational stage, and CD spectra are (automatically) acquired at many different angles.

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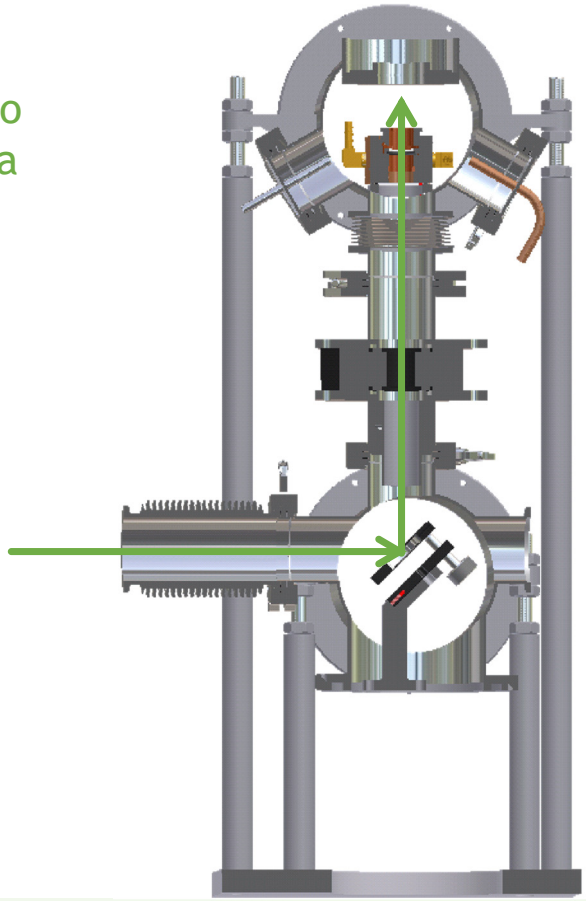
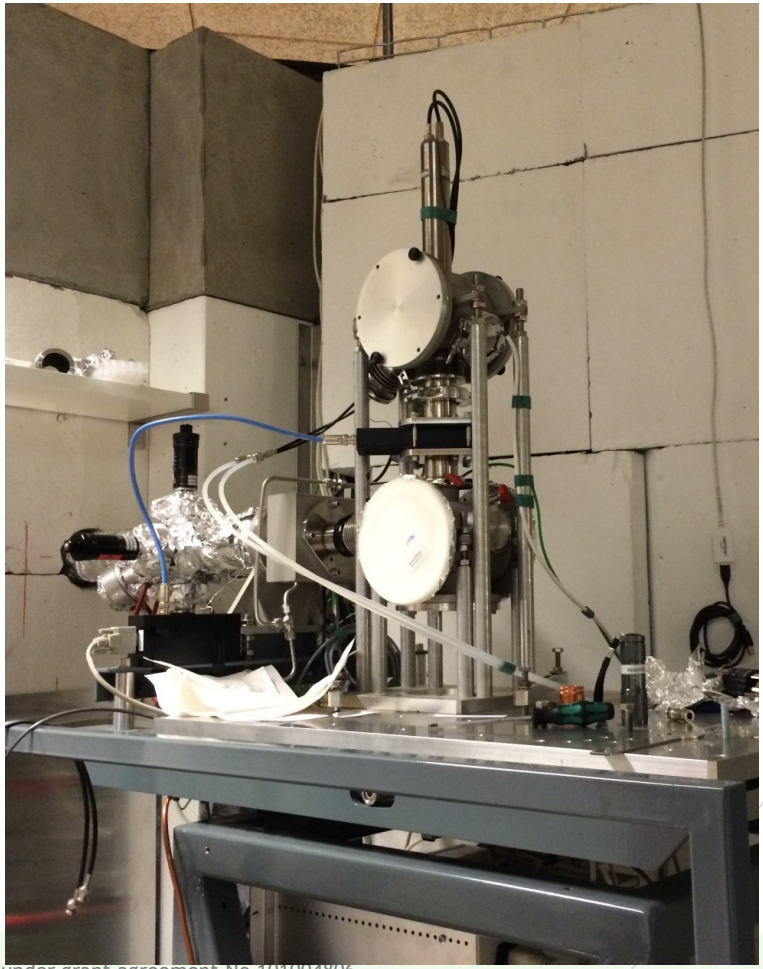
Measurement options…

Periscope

The periscope allows samples to be measured in a horizontal position





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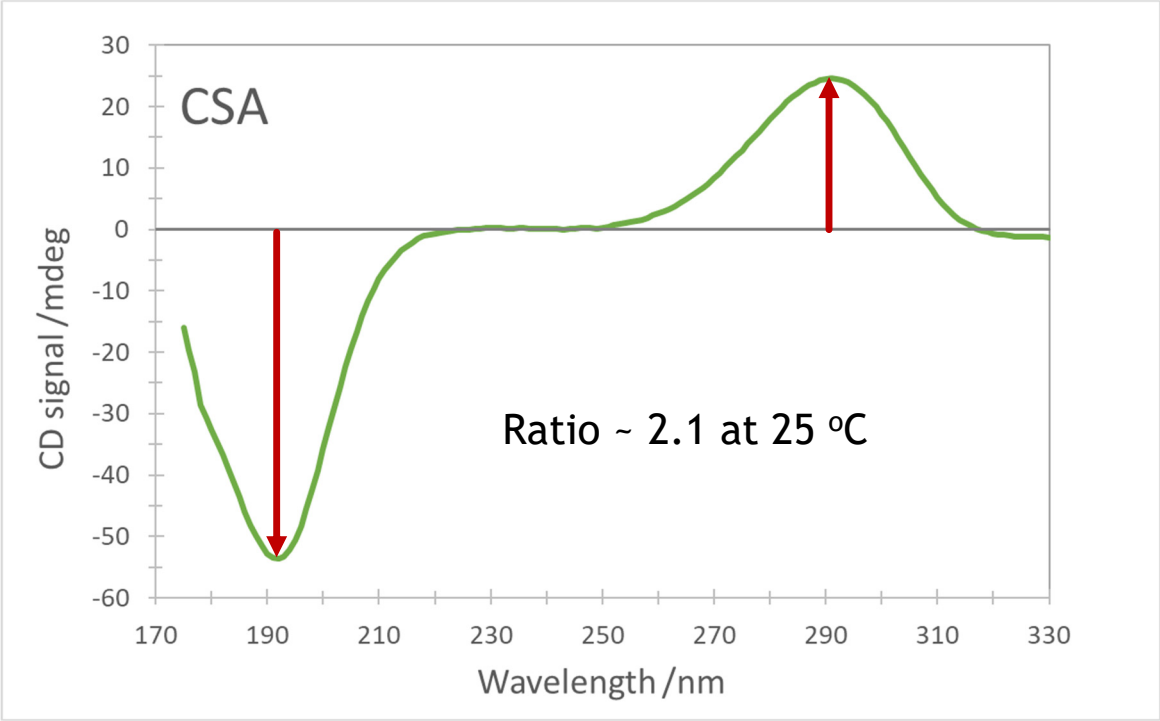
Calibration and instrument health

As with any other instrument, it is vital that you keep your instrument well calibrated

Check the calibration at the start of each day using (1S)-(+)-10-camphorsulfonic acid (CSA)



$\Delta\varepsilon_{290.5nm}$ = 2.36 $M^{-1}cm^{-1}$

MW = 232.30 g/mol

Use ~7 mg/ml in 0.1 mm cell

CSA concentration:

- CSA is hydroscopic
- Measure A_{285nm} in 1 cm cuvette
- Use ε_{285} = 34.6 $M^{-1}cm^{-1}$
- Rule of thumb:

  A_{285}(1cm) = 1 for 6.71 mg/ml

Ratio must be above 2. Ratio is very temperature dependent: always use 25°C.

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Calibration and instrument health

With time any spectrophotometer may develop stray light

- Stray light may pass through the sample
- If the absorption is high, the stray light hitting the detector may be significant
- The HT will become too low when keeping DC bias constant
    - Leads to CD features being measured too low!

The absorbance at the 192 nm CSA peak is relatively high

➢ Stray light shows up as the 192 / 290 ratio becoming low

Daily measurements of the CSA peak values gives a track record of the health of the instrument:

- The 192 / 290 ratio gives information about stay light changes over time
- The value of the 290 nm CSA CD signal gives information about the absolute calibration

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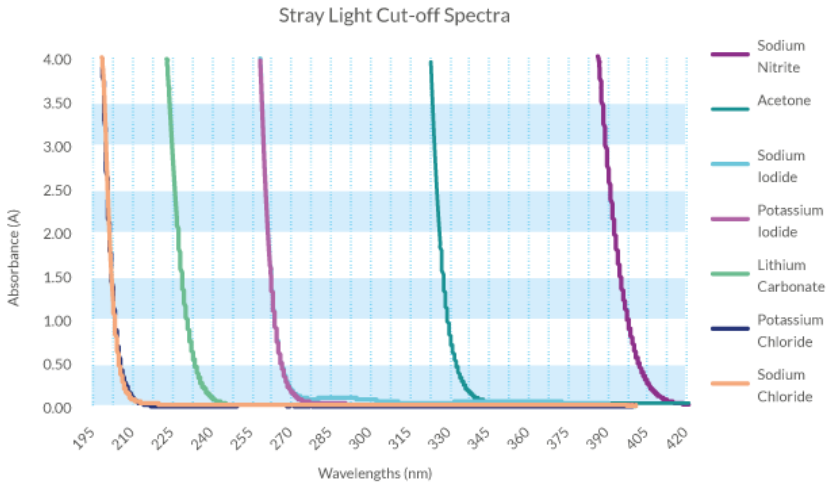
Calibration and instrument health

With time any spectrophotometer may develop stray light

E.g. Starna has reference salts for measuring stray light



Or you can make your own 12 g/l KCl solution:

- Absorbance > 2.0 measured at 198 nm against a water reference

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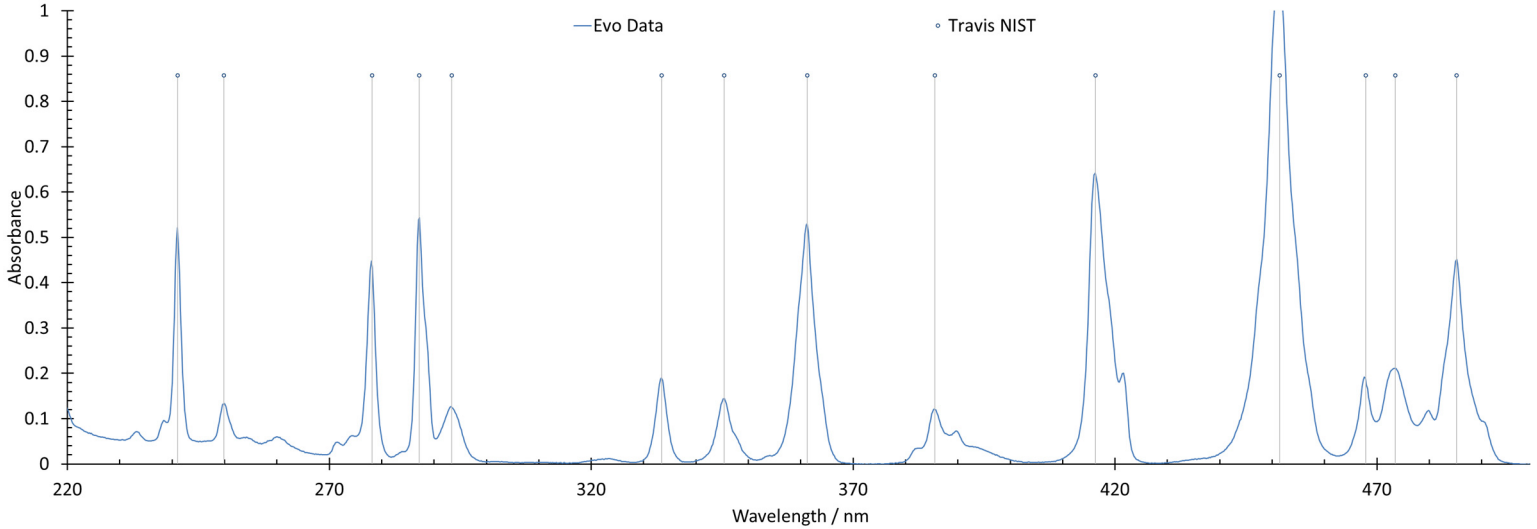
Calibration and instrument health

Check the wavelength calibration

- Daily by checking the position of the two CSA peaks (a rough estimate)
- Every few months using Holmium Oxide (HoOx)
  - 40 g/L solution of holmium oxide in 10% (v/v) perchloric acid
  - Store in sealed 10 mm cuvette



Calibration data are found in:

J.C. Travis *et al*. J. Phys. Chem. Ref. Data 34, 2005, 41–56

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